Ollscoil na hÉireann, Corcaigh
National University of Ireland, Cork

Department of Anatomy & Neuroscience
Head of Dept. John F. Cryan

Novel insights into the role of hippocampal TLX in neurogenesis, neuroinflammation and behaviour in adolescence and adulthood

Thesis presented by

Danka Atanasova Kozareva

under the supervision of

Dr. Yvonne M. Nolan

Prof. John F. Cryan

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Declaration

All work presented in this thesis is original and my own and has not been submitted for another degree, either at University College Cork or elsewhere.

Danka Atanasova Kozareva

October 2018

Author contributions

All the work conducted in this thesis was performed independently by the author except for the following contributions:

Chapter 2

Dr. Heuston and Dr. Ó'Léime assisted with genotyping and maintenance of the mouse colony. Ms. Crotty provided invaluable insight into stereology analysis and imaging techniques. All authors listed helped prepare the manuscript for publication.

Chapter 3

Dr. Moloney and Dr. Hoban assisted with primer design and data analysis. Dr. Rossini was responsible for the breeding and maintenance of the $CX3CR1^{KO}$ mouse colony. All authors listed helped prepare the manuscript for publication.

Chapter 4

All authors listed helped prepare the manuscript for publication.

Chapter 5

Dr. J O'Leary helped conduct the behavioural testing. Dr. Hueston established the transgenic mouse colony and helped maintain it throughout the study. All authors listed helped prepare the manuscript for publication.

Chapter 6

Ms. Foley assisted with stereotaxic surgeries and behavioural testing. All authors listed helped prepare the manuscript for publication.

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Abstracts and publications arising from this work

Published articles:

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Kozareva, D.A., Moloney, G.M., Hoban, A.E., Rossini, V., Nally, K., Cryan, J.F., Nolan, Y.M. A role for the orphan nuclear receptor TLX in the interaction between neural precursor cells and microglia. *Neuronal Signalling.* 3 (1): NS20180177. doi: 10.1042/NS20180177

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Kozareva, D.A., Hueston, C.M., Ó'Léime, C.S., Cryan, J.F., Nolan, Y.M. (2015). TLX, a regulator of neural stem cell self-renewal, is required for microglial integrity in the adult rodent dentate gyrus. Poster, CINP Thematic Meeting on Stress, Inflammation and Depression: focus on novel antidepressant targets. June 4th – 6th; Trinity College Dublin, Ireland.

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Abbreviations

5-hmc: 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine
5-HT: Serotonin or 5-hydroxytryptamine
5-HT$_{1A}$: serotonin 1A receptor
AAV: adeno-associated viral
Ach: Acetylcholine
ADHD: attention deficit hyperactivity disorder
Akt: Protein kinase B
AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA: One-way analysis of variance
Arc: activity-regulated cytoskeleton-associated protein
Arg1: Arginase1
Aqp4*:* Aquaporin 4
Ascl1: achaete-scute homolog 1
BBB: blood-brain barrier
Bcl-2: B-cell lymphoma 2
BCL11A: B-cell lymphoma/leukemia 11A
BDNF: brain-derived neurotrophic factor
bHLH: basic helix-loop-helix
BLBP: brain lipid binding protein
BMP: bone morphogenic protein
BMP4: Bone morphogenic protein 4
BMPR1: Bone morphogenic protein receptor, type 1
BMPR2: Bone morphogenic protein receptor, type 2
BOLD: blood oxygen level dependent
BrdU: 5-bromo-2'-deoxyuridine
CA: Cornu ammonis
cAMP: cyclic adenosine monophosphate
CD45: lymphocyte common antigen
CD68: cluster of differentiation 68
CD206: C-Type Mannose Receptor 1
CD200: OX-2 membrane glycoprotein, cluster of differentiation 200
CD200R: Cell surface transmembrane glycoprotein CD200 receptor 1
C. elegans: *Caenorhabditis elegans (C. elegans)*
ChIP: chromatin immunoprecipitation
Cl$^-$: chloride
[Cl$^-$]$_i$: intracellular concentration of chloride
CNS: central nervous system
CoREST: REST (RE1-silencing transcription factor) corepressor 1
COX-2: cyclooxygenase 2
CREB: cAMP response element binding protein
CS: conditioned stimulus
CSF1R: colony-stimulating factor 1 receptor
CX3CL1: Fractalkine/ chemokine (C-X3-C motif) ligand 1
CX3CR1: Chemokine receptor 1
DCX: Doublecortin
DG: dentate gyrus
dDG: dorsal dentate gyrus
DNA: deoxyribonucleic acid
DNE: dentate neuroepithelium
E: embryonic day
ECT: electroconvulsive treatment
EGF: epidermal growth factor
EPM: elevated plus maze
F4/80: EGF-like module-containing mucin-like hormone receptor-like 1
FGF-2: basic fibroblast growth facto
FGFR2: FGF-2 receptor
fMRI: functional magnetic resonance imaging
FMRP or FMR1: Fragile X mental retardation protein
FST: forced swim test
FXR2: autosomal paralog Fragile X mental retardation syndrome-related protein 2
FZD: seven-pass transmembrane receptors of the Frizzled family
GABA: γ-aminobutyric acid
GADD45β: Growth arrest and DNA-damage-inducible protein 45 β
GCL: granule cell layer
GFAP: glial fibrillary acidic protein
GFP: green fluorescent protein

Gli1: glioma-associated oncogene
GRs: glucocorticoid receptors
GSH2: Glutathione synthetase
GSK-3β: glycogen synthase kinase 3 beta
GWAS: genome-wide association study
H&E: Haemotoxylin and Eosin
H3K4: trimethylation of histone H3 at lysine 4
HATs: histone acetylases
HDACs: Histone deacetylases
HDAC1: Histone deacetylase 1
HDAC2: Histone deacetylase 2
HDAC5: histone deacetylase 5
Hes1: hairy and enhancer of split-1
HIV-1: human immunodeficiency virus type 1
Iba-1: Ionized calcium binding adaptor molecule 1
i.c.v.: intracerebroventricular
Id: Inhibitor of DNA-binding/differentiation protein
Id2: DNA-binding protein inhibitor 2
IFNγ: interferon gamma
IGF-1: insulin-like growth factor 1
IGF-2: insulin-like growth factor 2
IHC: immunohistochemistry
IL-1β: Interleukin-1 beta
IL-1R1: interleukin 1 receptor, type I
IL-6: interleukin 6
IL-10: interleukin 10
iNOS: inducible isoform nitric oxide synthases
i.p.: intraperitoneal
iPSCs: induced pluripotent stem cells
IQ: Intelligence quotient
JNK: Jun N-terminal kinase
Let-7: lethal 7 microRNA
LPS: Lipopolysaccharides
LSD1: Lysine-specific histone demethylase 1A
LTP: long-term potentiation
LV^{SCR}: lentivirus with scrambled shRNA sequence
LV^{TLXShRNA}: lentivirus carrying shRNA to silence TLX
Mash1: see Ascl1
MBD1: Methyl-CpG-binding domain protein 1
Me: methylation
MeCP2: Methyl-CpG-binding protein 2
MeCP2^{KO}: MeCP2 knockout
mGluR5: metabotropic glutamate receptor 5
MHC I: major histocompatibility complex class I
MHCII: major histocompatibility complex class II
miRNA: MicroRNA
MMP2: matrix metallopeptidase 2
mPFC: medial prefrontal cortex
MRI: magnetic resonance imaging
mRNA: messenger RNA
MWM: Morris water maze
NDS: normal donkey serum
Ne: Noradrenaline
NEC: neural epithelial cells
NeuN: neuronal nuclei
NeuroD1: neurogenic differentiation 1
NeuroG2: Neurogenin-2
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
NGS: normal goat serum
NICD: Notch intracellular domain
NMDAR: N-methyl-D-aspartate receptor
NOR: novel object recognition
NPC: neural precursor cell
NR1: Nuclear receptor 1
NR2B: Glutamate [NMDA] receptor subunit epsilon-2, also known as N-methyl D-aspartate receptor subtype 2B
Nr2e1/**TLX**: Tailless homolog orphan nuclear receptor subfamily 2 group E member 1
NSC: neural stem cell
NT-3: neurotrophin 3
NT-4: neurotrophin-4
NUMBL: NUMB like, endocytic adaptor protein
Oct3/4: octamer-binding transcription factor 4
OF: open field
P: postnatal day

p: phosphorylation
p21: cyclin-dependent kinase inhibitor 1
p53: tumor protein TP53
p57: cyclin-dependent kinase inhibitor 1C
P63: tumour protein p63
P75NTR: p75 neurotrophin receptor
Pax2: Paired box gene 2
Pax6: paired-box protein 6
PBS: phosphate buffered saline
PCP: planar cell polarity
PD: Parkinson's disease
PFA: paraformaldehyde
PFC: prefrontal cortex
PI-3 kinase: phosphoinositide 3-kinase
Plce1: 1-Phosphatidylinositol-4,5-bisphosphate phosphodiesterase epsilon-1
ppargc1b: a gene that transcriptionally regulates oxidative energy metabolism by cells
PROX1: prospero homeobox protein 1
PSA-NCAM: polysialylated neural cell adhesion molecule
PSD-95: postsynaptic density protein 95
PTCH1: protein patched homolog 1
pten: phosphatase and tensin homolog
Ras/**Raf**/**MEK**/**ERK**: family of proteins that belong to the class of small GTPases (Ras) / rapidly accelerated fibrosarcoma (Raf)/ mitogen activated protein kinase kinase (MEK)/ Extracellular signal-regulated kinase (ERK)
RBPJ: Recombinant binding protein suppressor of hairless
RGC: radial glial cell
RNA: ribonucleic acid
ROS: reactive oxygen species
RPC: retinal progenitor cells
S100β: astrocyte-specific marker calcium binding protein β
SD: Sprague Dawley
SGZ: subgranular zone
Shh: Sonic Hedgehog
Sirt1: Sirtuin 1
SMAD4: Mothers against decapentaplegic homolog 4;
SMADS: homologies to the Caenorhabditis elegans SMA ("small" worm phenotype) and Drosophila MAD ("Mothers Against Decapentaplegic") family of genes
SMO: protein Smoothened
Sox2: sex-determining region Y- box2
SVZ: subventricular zone
Tbr1: T-box brain 1
Tbr2: T-box transcription factor Eomes
TET1: Ten-eleven translocation methylcytosine dioxygenase 1
TGFβ: transforming growth factor beta
TLRs: toll-like receptors
TLX: Protein Tailleess Homolog; the human homologue of the Drosophila *tailless* gene
TNFα: Tumor necrosis factor alpha
TrkB: Tropomyosin receptor kinase B
TrkC: Tropomyosin receptor kinase C
US: unconditioned stimulus
vDG: ventral dentate gyrus
VEGF: vascular endothelial growth factor
VGF: vgf nerve growth factor inducible
VHL: von Hippel-Lindau disease tumour suppressor
VPA: valproic acid
VSV-G: vesicular stomatitis virus glycoprotein
WT: wildtype

Abstract

The orphan nuclear receptor TLX is a key regulator of embryonic and adult neurogenesis and is primarily expressed in the neurogenic niches of the brain. Adult hippocampal neurogenesis is characterized by the generation of new granule cells that become integrated into the circuitry and contribute to cognitive function. Impaired hippocampal neurogenesis has been reported in neurodegenerative and psychiatric conditions and efforts to develop therapeutic strategies that employ the hippocampal NSCs are ongoing. TLX is one of an array of intrinsic factors regulating NSC proliferation and differentiation, in combination with extrinsic regulators such as stress, exercise and neuroinflammation. Adolescence is a sensitive period of neurodevelopment, during which the environment can have profound effects. Little has been reported on TLX function during adolescence. TLX performs its role by transcriptional activation and repression of a number of genes in order to promote NPC proliferation and to maintain the neurogenic pool of NSCs in the DG. In its absence neurogenesis is dramatically decreased, positioning it as the master modulator of the neurogenic process.

The aims of this thesis were to investigate the role of the immune cells of the brain, microglia, on hippocampal neurogenesis in the presence/absence of TLX, and to determine whether TLX plays a role in microglia-neuronal crosstalk; to investigate the role of TLX in hippocampal neurogenesis during adolescence, and the impact thereupon of exercise and stress; and to understand the role of TLX in behavioural and cognitive function during adolescence and adulthood, through evaluating two deletion

models of TLX – a spontaneous deletion mouse model, and a rat model using lentiviral knockdown of TLX. We have shown that a lack of TLX is implicated in the deregulation of microglial phenotype, resulting in activated microglia and elevated levels of the pro-inflammatory cytokine IL-1β, and that consequently the survival and function of newborn cells in the hippocampus is impaired. Furthermore, we showed that when neuronal-microglial signalling is impaired in the absence of the chemokine receptor CX3CR1, the expression of TLX and some of its downstream targets is altered. We also have shown that TLX is necessary for the pro-neurogenic effects of exercise during adolescence, and that deletion of TLX modulates motor, cognitive and anxiety-related behaviours during adolescence and adulthood in both male and female mice. Lastly, we demonstrated that silencing of TLX expression in the dDG during adolescence resulted in impairments in hippocampal-independent behaviours, which either did not persist or were reversed during adulthood.

In summary, we confirm the importance of TLX in the regulation of neurogenesis and neuronal-microglial cross-talk as well as the temporal importance and function of TLX during adolescent development. Disentangling the complex interactions between TLX, the immune system and other extrinsic regulators of hippocampal neurogenesis would thus provide valuable insights into the development of therapeutics for neurodegenerative disorders using stem-cell-stimulation based approaches.

Chapter 1

General Introduction

1.1 The Hippocampus

1.1.1 Anatomy of the hippocampus

The hippocampus (derived from the Greek *hippocampus*: *hippos*, meaning “horse”, and *kampos*, meaning “sea monster”) is a deep brain structure and part of the limbic system. The remarkable resemblance of the hippocampus to a seahorse is evident from a dissection of the human hippocampus (See Figure 1.1 (Judas and Pletikos, 2010)). The elongated curved shape is preserved across all mammalian species and runs bilaterally along a dorsal (septal)-to-ventral (temporal) axis in rodents or posterior-to-anterior axis in humans in the medial temporal lobe of the brain (Strange et al., 2014).

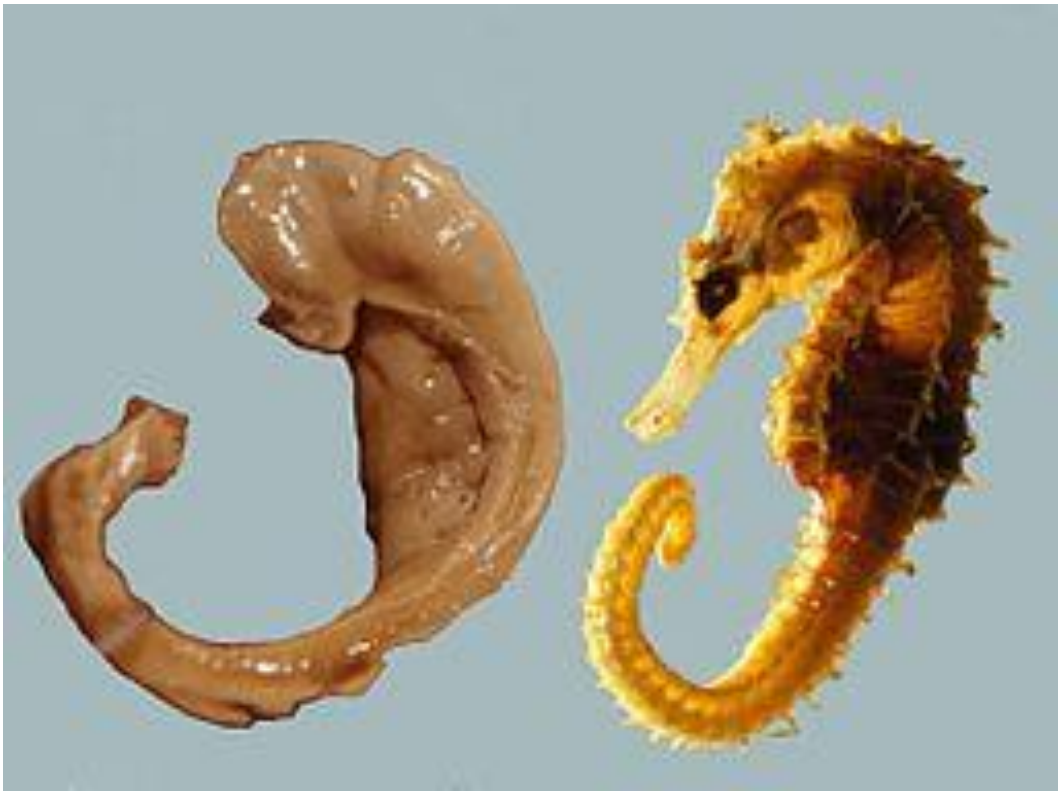

Figure 1.1: Hippocampus and seahorse by Prof. Laszlo Seress, University of Pècs, Hungary, licensed under CC-BY-SA.

The hippocampal formation consists of three separate subregions – the dentate gyrus (DG), the hippocampus proper or the cornu ammonis (comprised of CA3, CA2 and CA1) and the subiculum (van Strien et al., 2009). The DG lies in the medial-most part of the cerebral cortex and is separated ventrally from the CA1 and subiculum by the hippocampal fissure (See Figure 1.2). This C-shaped trilaminate region consists of the granular cell layer (GCL), the molecular layer and the polymorphic cell layer, also known as the hilus (Schultz and Engelhardt, 2014). The cell bodies of the granule cells, the principal cell type within the DG, are compactly held within the GCL and are as numerous as ~ 1.2 x 10^6 in the rodent and ~ 15 x 10^6 in the human DG (Amaral et al., 2007). The apical spiny dendrites of these cells branch extensively into the molecular layer of the DG, where they receive afferent connections (Schultz and Engelhardt, 2014). Characteristic myelinated axons arise from the granule cells and extend through the hilus until they terminate in large synapses on the pyramidal cells of CA3. Ramon y Cajal called these axons mossy fibres and they are known to create as many as 40 synapses within one excrescence. The DG also receives input from the entorhinal cortex through the perforant pathway (See Figure 1.2). The deepest layer of the DG – the hilus – comprises of a mixture of afferent and efferent fibres and interneurons (See Figure 1.2 (Amaral et al., 2007; van Strien et al., 2009)). Adjacent to the hilus is the CA3 area of the hippocampus proper, which receives input not only from the granule cells of the DG, but also from cells in the entorhinal cortex via the perforant pathway. CA3 consists of one cellular layer, the pyramidal cell layer, which can be divided in several sublayers from superficial to deep: stratum lacunosum-moleculare, comprising the apical tufts of the apical dendrites where the entorhinal connections of the perforant path terminate; stratum radiatum, containing the basal branches of these dendrites

where the mossy fibres of the DG granule cells terminate, after passing through and forming synapses in the stratum lucidum; stratum pyramidale is the pyramidal soma layer of the CA3, next to which is the stratum oriens containing the recurrent collaterals (Schultz and Engelhardt, 2014; van Strien et al., 2009). The anatomical organization of CA2 and CA1 is identical to CA3 with the exception of a missing stratum lucidum sublayer. CA2 is significantly smaller than CA3 and CA1 and is located between the two, receiving input from layer II of the entorhinal cortex via the perforant pathway and from CA3 via the Schaffer collaterals (See Figure 1.2). CA1 receives input from CA3 via the same collaterals and these projections form important part of memory formation and of the hippocampal trisynaptic loop. Furthermore, CA1 is the source of greatest hippocampal output pathways, one of which terminates in layer V of the entorhinal cortex, while the other one travels to the subiculum (Cuperlier et al., 2007; Schultz and Engelhardt, 2014). The subiculum overlaps with CA1 and forms the interface between the hippocampus and the entorhinal cortex (See Figure 1.2). The trilaminate architecture of the subiculum consists of the molecular layer, where apical dendrites extend from the subiculum to CA1 (See Figure 1.2); the enlarged pyramidal cell layer containing the soma of subiculum neurons and a deep polymorphic layer (O'Mara, 2005; Schultz and Engelhardt, 2014). Besides CA1, the subiculum also receives input from layer III of the entorhinal cortex. Being the main output of the hippocampus, subiculum pyramidal neurons send projections to a number of areas among which are the nucleus accumbens, septum, prefrontal cortex (PFC) and entorhinal cortex. Additionally, the subiculum has been said to contain “direction” and “place” cells which are pyramidal neurons with spatially restricted firing patterns (O'Mara, 2005; Taube et al., 1990). The entorhinal cortex is the major

relay area between the hippocampus and the neocortex. It is now primarily identified based on connectivity to the hippocampus (Canto et al., 2008; Krimer et al., 1997).

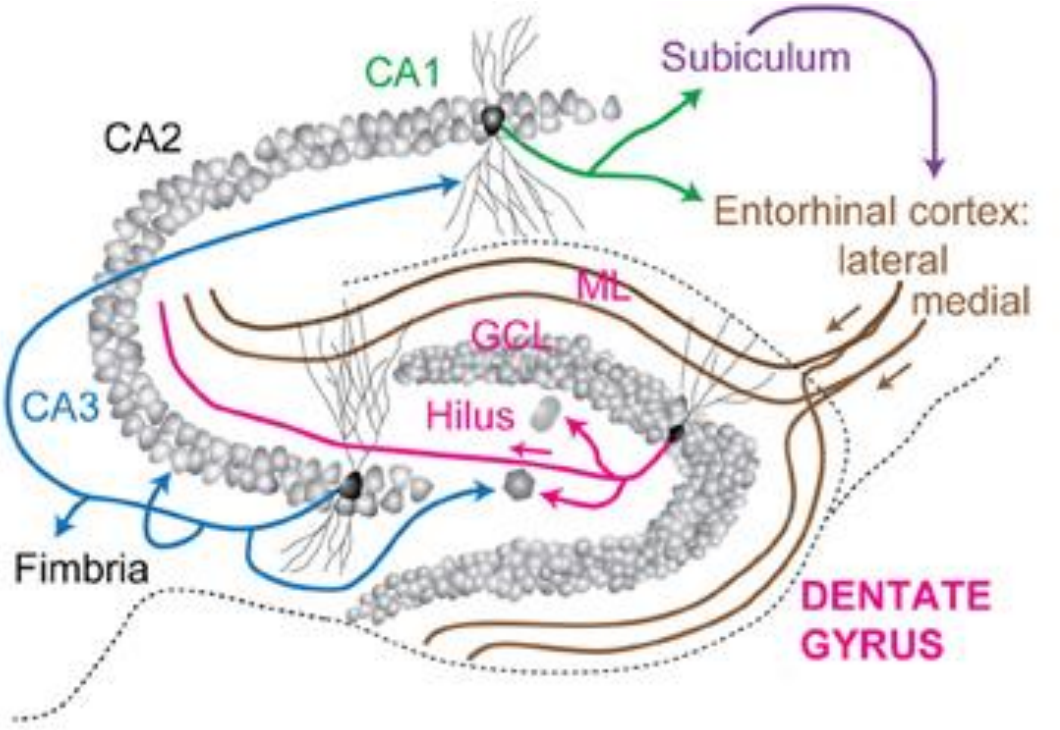

Figure 1.2: Hippocampal & Dentate Gyrus Anatomy of the rodent. Arrows represent excitatory aonal projections. Adapted from (Piatti et al., 2013).

1.1.2 Function of the hippocampus

Functionally, the hippocampal formation is involved in memory consolidation, processing of emotional stimuli, spatial navigation and orientation and learning (Kandel, 2000). The pioneering evidence linking the hippocampus with memory consolidation and learning new information came from a case study of patient H.M. in the 1950s that revolutionized memory research. Due to continuous suffering from epileptic seizures, the patient underwent bilateral medial temporal lobectomy, in order to remove the hippocampus and neighbouring parahippocampal regions. The surgery relieved the symptoms of epilepsy but resulted in severe anterograde amnesia, or the

ability to create new memories, and temporally graded retrograde amnesia, or the ability to recall memories that were encoded just before the surgery (Scoville and Milner, 1957). This surgery provided strong evidence of a role for the hippocampus in memory formation. More recent studies of patients with bilateral damage confined to the hippocampal region report that new learning (anterograde amnesia) and encoding of information assimilated prior to the occurrence of the damage (retrograde amnesia) were severely impaired (Bayley et al., 2006; Manns et al., 2003).

The discovery of how the hippocampal formation underpins spatial location and plays role in navigation and orientation brought the Nobel Prize in Physiology and Medicine in 2014 to Prof. John O'Keefe for his discovery of "place cells" (O'Keefe, 1976; O'Keefe and Dostrovsky, 1971) and to Prof. May-Britt Moser and Prof. Edvard I. Moser for their discovery of the "grid cells" in the entorhinal cortex. The circuitry which these cells form with place cells in the hippocampus is known as the inner GPS of the brain (Fyhn et al., 2004; Hafting et al., 2005; Sargolini et al., 2006). In effect, specific cells in the hippocampal formation fire action potentials in response to a rodent's current location associated with their position in relation to the external environment (allocentric) rather than to the body (egocentric) and thus the cognitive map is formed (Hartley et al., 2014). As such, it provides a long-term memory containing information about the spatial relationships between objects and places, paths that connect them and possible rewards or dangers that can be encountered on each of these paths (1999; Burgess et al., 2001). The main evolutionary advantage of the cognitive map is that an animal can go about familiar environments without

continuously creating new representations of them (Hartley et al., 2014). Support for the cognitive map theory and the involvement of the human hippocampus in navigation and spatial orientation comes from studies examining the performance of patients with unilateral damage to the hippocampal formation due to intractable epilepsy on the Nine-Box Maze, which relies on spatial-, object-, working-, and reference- memory, where patients displayed significant spatial and working memory deficits compared to controls (Abrahams et al., 1999; Abrahams et al., 1997). Additionally, research in patients with temporal lobe lesions in which their ability to orienteer in a novel urban area presented as videotape material (Maguire et al., 1996), as well as studies of healthy controls whose brain activity by functional magnetic resonance imaging (fMRI) was assessed while they navigated through a complex virtual reality town (Maguire et al., 1998), have all shown strong correlation between hippocampal activation and representation of places and best path of navigation to them (reviewed by (Maguire et al., 1999)). Results from a study where tasks examining context-dependent episodic memory and tasks tapping into ability to remember allocentric spatial information were dissociated in a patient with bilateral hippocampal pathology were consistent with previous findings. Collectively these data illustrated the importance of the hippocampal formation in the ability to successfully navigate and recall spatial memories (Spiers et al., 2001). For a comprehensive review of the literature on the involvement of human hippocampus in spatial memory and the cognitive map see (Burgess et al., 2002).

Rodent studies have further elucidated the involvement of the hippocampus in memory and learning, while also unveiling the involvement of different cortical areas in post-consolidation memory processing (reviewed by (McKenzie and Eichenbaum, 2011); (Frankland et al., 2004; Ross and Eichenbaum, 2006)). Furthermore, a lesion study in rats has shown that intact hippocampus and subiculum are necessary for the acquisition of a non-spatial task as well as for the subsequent retrieval of long-term memory (Clark et al., 2002). Evidence for a potential mechanism of consolidation, for spatial memories specifically, comes from studies employing microelectrode array and electromyogram recordings of rodent brain activity, which have shown that the sequence and pattern of cells firing during the acquisition of a spatial task recur later during sleep or rest awake state, a phenomenon known as hippocampal "replay" (Ji and Wilson, 2007; Wilson and McNaughton, 1994). Preventing the occurrence of hippocampal replay by stimulation-induced suppression after learning a spatial task has been shown to impair recall of the task, while stimulation-induced suppression of hippocampal activity at a later stage had no effect on memory performance (Ego-Stengel and Wilson, 2010; Girardeau et al., 2009). Additionally, an independent group determined that spatial memory consolidation and subsequent performance could be predicted by hippocampal network activities during learning and later memory trace formation (Dupret et al., 2010).

In the 1930s the hippocampus, along with several other areas, was suggested to be a constitutive part of the "emotion system" in the brain. Since then it has been shown that the hippocampal formation plays an indirect role in emotional computation and

response in the brain (Kandel, 2000). For instance, a study examining the fMRI BOLD (blood oxygen level dependent) response of participants whilst they were presented with various documentary-style narratives showing people in different real-life circumstances, illustrated that the hippocampus facilitates appropriate emotional responses when processing social emotions. The authors explained their finding with the fact that processing emotional states of others' calls for complex inferences of others' mental states based on consideration of the contextual information and personal qualities, which involves hippocampal and medial prefrontal cortex circuitry (mPFC) (Immordino-Yang and Singh, 2013). Additionally, animal and human studies have shown strong associations between hippocampal and mPFC neuronal connectivity and the regulation of emotions, implicating that impairments in the stability of these circuits may be the basis of the pathophysiology of anxiety-related psychiatric conditions as well as schizophrenia and post-traumatic stress disorder (reviewed by (Jin and Maren, 2015)). Furthermore, a series of investigations have established that in emotional situations, the interaction between amygdala and hippocampus produces strengthening of encoding and storage of emotional stimuli, and that the hippocampal formation can impact upon the activation of the amygdala once the organism is confronted with these stimuli again (reviewed by (Phelps, 2004)). Another link between emotional processes and hippocampal activity comes from studies, both rodent and human, showing that stressful experiences and chronic stress can have dramatic effects on hippocampal-dependent memory performance and cognitive function as well as lead to reduction in hippocampal volume (reviewed by (Lupien et al., 2009)). The reduction in hippocampal volume was hypothesized to be caused by neuronal cell loss caused by cell death (McEwen and Sapolsky, 1995).

However, accumulating evidence from both rodent and human studies has revealed that the stress-induced decrease in hippocampal volume is accounted for by changes in neuronal morphology and suppressed neuronal proliferation (reviewed by (Kim et al., 2015a)). Finally, ample evidence from patients suffering from major depressive disorder has accumulated, illustrating that the hippocampal volume is reduced in depressive patients (Campbell et al., 2004; Frodl et al., 2004). Moreover, hippocampal volume increases in a time- and dose- dependent manner with antidepressant treatment and that hippocampal atrophy correlates with anxiety-related symptoms (Rusch et al., 2001; Sheline et al., 2003).

Reconciling this variety of hippocampal functions occurred in the late 1990s with Moser's theory hypothesizing that the hippocampus may not act as a singular structure, but rather be segregated across its septo-temporal axis with distinct functions being regulated by either the dorsal or ventral part (Moser and Moser, 1998). The theory was postulated on the following 3 observations: there are distinct anatomical input/output connections for the dorsal and ventral hippocampus (Swanson and Cowan, 1977), dorsal hippocampal lesions severely disrupted spatial learning in the Morris water maze tasks (MWM), while lesions of similar size in the ventral hippocampus produced no impairments (Moser et al., 1993), the stress response and emotional behaviour of adult rats was altered in response to ventral, but not dorsal, hippocampal lesions (Henke, 1990). Up to date, there is substantial number of studies supporting Moser's theory. Cytotoxic lesion studies in rodents revealed a dissociation between dorsal and ventral hippocampal function, such that dorsal but not ventral lesions impaired

performance on spatial learning tasks different from the MWM task, such as appetitive maze tasks (T-maze rewarded alternation) and radial maze tasks. Ventral but not dorsal hippocampal lesions led to altered performance in contextual and cued fear conditioning tasks (reviewed by (Bannerman et al., 2004)). Additionally, dorsal hippocampal lesions as well as pharmacological inhibition of function were shown to impair paired associate learning in a touchscreen task in mice (Kim et al., 2015). Studies employing alternative methods for segregated regional loss of function, such as temporary pharmacological manipulation through intracerebral drug microinfusions have replicated the findings from lesion experiments, illustrating again that disturbing normal function in the ventral hippocampus results in decrease in fear and anxiety while the dorsal hippocampus is preferentially involved in spatial learning and memory (reviewed by (Bannerman et al., 2004; Fanselow and Dong, 2010)). Interestingly, structural MRI of the brains of London taxi drivers, subjects with considerable navigation experience and skills, revealed that their posterior (dorsal in rodents) region of the hippocampus is significantly enlarged compared to controls (Maguire et al., 2000). Gene expression data also support a segregation of the hippocampus along its septo-temporal axis (Fanselow and Dong, 2010). Notably, rather than a clear cut border between the dorsal and ventral region, more recently it has been suggested that the segregation is gradual, with functionally distinct region between the dorsal and ventral horns, namely the intermediate hippocampus (Fanselow and Dong, 2010), however, future studies need to better define this gradual anatomical gradient functionally and molecularly.

1.2 Hippocampal neurogenesis across the life span

The process of generating functional neurons from stem and precursor cells in the central nervous system (CNS) was originally believed to occur strictly during embryonic and early postnatal development in mammals. A century ago this idea was challenged with the discovery of neurogenesis in the adult brain. Ezra Allen was the first to demonstrate that mitosis persisted in the lateral walls of adult albino rats (Allen, 1918). Several decades later, Altman and Das followed up this research and determined that neurogenesis occurred in the adult rat and guinea pig hippocampus (Altman and Das, 1965b). Since then, evidence began to accumulate in favour of the existence of this process in the human brain also. It was reported that the rate of proliferation and the process of functional integration of adult born neurons into existing circuitry was remarkably similar across species (Eriksson et al., 1998). Additionally, a study measuring the concentration of nuclear bomb test-derived ^{14}C in genomic deoxyribonucleic acid (DNA), found evidence for the birth of as many as 700 new neurons each day in the human hippocampus (Spalding et al., 2013). Nonetheless, the controversy over the existence of human hippocampal neurogenesis has been raised once more (Lee and Thuret, 2018; Snyder, 2018). In a recent investigation of postmortem brain tissue obtained from 18 adults and 19 perinatal and postnatal samples (age range: 14 gestational weeks to 77 years), it was reported that no newborn neurons were found in the DG of adults and only a few isolated young neurons were observed in samples from young individuals (7 to 13 years of age). The samples with the most numerous immature neurons observed came from perinatal and postnatal (up to 1 year of age) tissue (Sorrells et al., 2018). On the contrary, using a similar immunohistochemical approach, another group of researchers observed immature and

mature adult born neurons in the hippocampal samples obtained postmortem from 28 healthy individuals (age range: 14 to 79 years of age) and the number of each cell type was estimated to be at least in the thousands (Boldrini et al., 2018). It is possible that the big discrepancy in results stems from the fact that in the former study, tissue was obtained from individuals suffering a wide range of diseases (although full medical history was not provided), while in the latter study tissue was obtained from healthy individuals (Snyder, 2018). Given the similarity in methods employed, both studies clearly demonstrate the limitations and caveats in studying neurogenesis in human postmortem tissue. To address more actively the existence of the phenomenon in the human brain, future studies need to examine not only evidence on an immunohistochemical level but also on a transcriptomic and gene expression level.

To date, two neurogenic niches have been identified in the adult mammalian brain, namely the subependymal neurogenic niche of the subventricular zone (SVZ), and the subgranular zone (SGZ) of the DG (Bond et al., 2015). The SGZ of the hippocampus is the focus of the present work. The putative hippocampal precursors are multipotent and divide infrequently. Morphologically, they are similar to radial glia with a triangular cell body and a large apical process extending and forming multiple arborizations into the dentate granule cell layer. The end processes of these stem cells terminate onto the vasculature. The progeny of SGZ stem cells migrate to the granule cell layer of the DG where they integrate into hippocampal circuitry as mature excitatory neurons (Gage, 2000). Immunohistochemical techniques allow for studying the newborn cells during the different phases of their development. For instance, the

intermediate filament nestin is used as a marker of neural stem cells, while immature neurons express the microtubule-associated protein doublecortin (DCX). Approximately four weeks after their birth, neurons have matured and begin to express NeuN instead of DCX. The thymidine analogue bromodeoxyuridine (BrdU) labels dividing cells and can be used in conjunction with the markers above for studying the proliferation, survival and differentiation fate of neural stem cells (NSCs (Zhang and Jiao, 2015); See Figure 1.3).

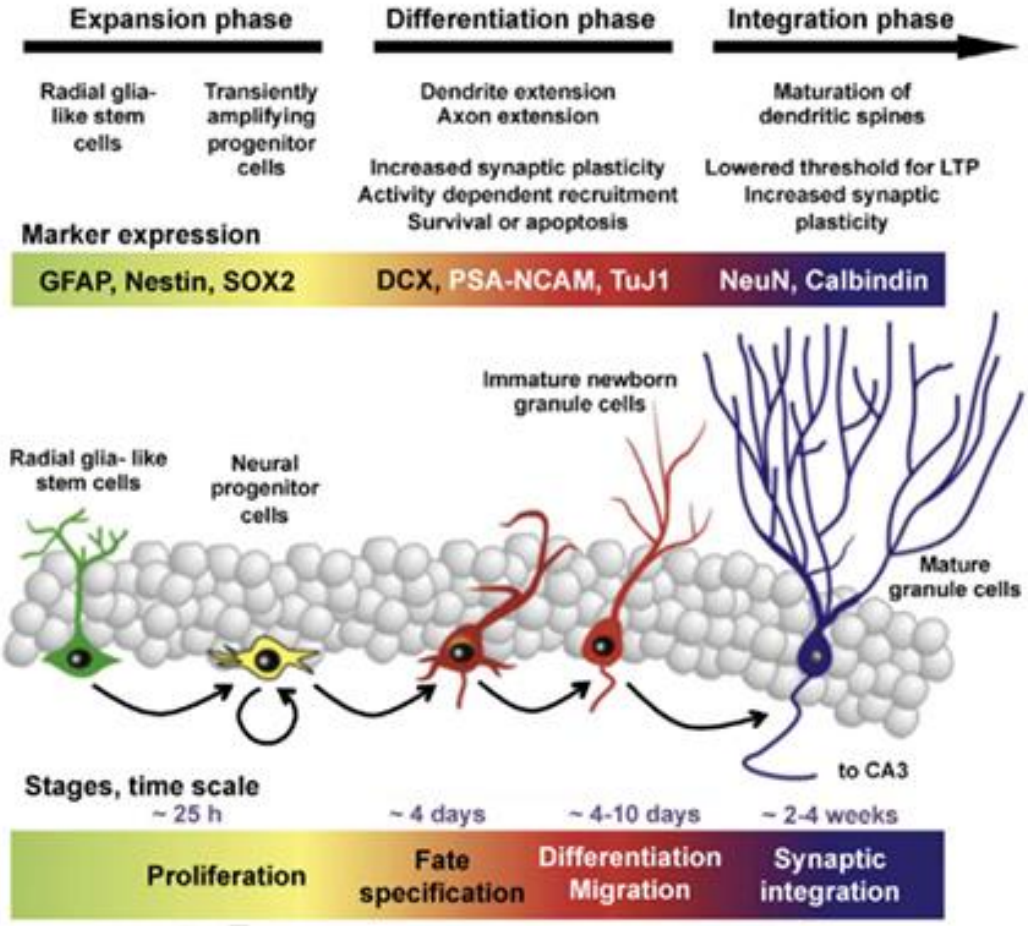

Figure 1.3: Stages of neurogenesis and markers of expression in the SGZ. Adapted from (Schouten et al., 2012).

1.2.1 Embryonic hippocampal neurogenesis

In the earliest stage of development, three distinct cell types comprise the embryo – the endoderm, from which the lining of most visceral organs arises; the mesoderm, from which all bones and skeleton muscles develop; and the ectoderm, which gives rise to the entire nervous system and the skin (Bear, 2007). The ectoderm is the outer most thin layer of the embryo and once it acquires the potential to form neural cells, the process of embryonic neurogenesis begins (Hartenstein and Stollewerk, 2015). For the single sheet of ectoderm to become neurogenic, the following two changes need to take place: firstly, a specialized area of the ectodermal tissue, known as the neural plate, forms a groove; secondly, the walls of this groove or the neural folds move close together, thereby shaping the neural tube (Semple et al., 2013). The brain and spinal cord develop from the neural tube, while the peripheral nervous system develops from the neural ectoderm lateral to the neural tube (Bear, 2007). In rodents, the formation of the neural tube is completed at approximately embryonic day (E) 9-9.5, which also marks the start of embryonic neurogenesis (Semple et al., 2013). Neural epithelial cells (NECs), considered as the earliest embryonic NSCs, divide asymmetrically at first, so that they expand their pool while also creating daughter neural progenitor cells (NPCs). Later after the neural tube is completely formed, NECs transform into radial glial cells (RGCs; (Zhang and Jiao, 2015)). RGCs also divide asymmetrically to maintain their population and give rise to neurons, glia and potentially other type of cells (Alvarez-Buylla et al., 2001). RGCs can produce neurons directly or indirectly through NPCs. NECs, NPCs, and RGCs can also divide symmetrically to increase their populations. By asymmetric division, NPCs renew themselves and produce a second daughter cell that either differentiates into a neuron immediately, or goes through a

fixed number of divisions before permanently differentiating into a neuron (Alvarez-Buylla et al., 2001; Hartenstein and Stollewerk, 2015). The embryonic stage-dependent NSC differentiation throughout development is depicted in Figure 1.4. RGCs also function as a scaffold through which the newborn neurons and NPCs migrate to their designated area directed by extracellular signals (Zhang and Jiao, 2015). In rodents, at approximately E15-E17.5, all neurons comprising the cortical and subcortical areas have been generated and have migrated, which is when RGCs differentiate into astrocytes and glia (Jin, 2016; Semple et al., 2013).

Interestingly, the development of the DG follows another unique path. A separate source of progenitor cells (also known as the dentate neuroepithelium; DNE) separates from the neural tube and migrates in close proximity to the pial surface (Urban and Guillemot, 2014). At E12.5 the hippocampal neuroepithelium and the cortical hem surround the DNE, and at E14.5 the DG becomes clearly distinguishable with dentate precursor cells migrating from the ventricular zone towards the pial surface of the cortex (Rickmann et al., 1987). RGCs from the hippocampal neuroepithelium produce hippocampal neurons and at E17.5 the hippocampal fissure is formed. The dentate precursor cells then begin to migrate and accumulate within the fissure. Some of these cells supposedly comprise the future layer of NSCs of the adult SGZ, while some produce neurons that form the GCL along with neurons produced by the hippocampal neuroepithelium progenitors of the ventricular zone (Urban and Guillemot, 2014). The typical V-shape of the DG, created by its two blades is contributed by cells produced by the cortical hem (Cajal-Retzius cells), which direct the migration of NPCs and the

organization of the hippocampal fissure (Del Rio et al., 1997). It is still contested whether the adult NSCs of the SGZ come from the DNE itself at around E13.5 (Seki et al., 2014; Seri et al., 2004), or whether they are generated perinatally within the ventral DG (vDG), migrate in late gestation to the dorsal DG (dDG), and become activated through Sonic Hedgehog (Shh)-signalling (Li et al., 2013). Thus, the origin of the adult NSCs may have important consequences to their function and regulation. Due to the lack of unique immunocytochemically detectable markers specific to NSCs as opposed to NPCs, embryonic NSCs are defined as multipotent self-renewing cells that have the capacity to populate a developing region of the CNS (Lee et al., 2010). Though not exclusive for embryonic NSCs, a large number of studies have identified nestin and SRY (sex determining region Y)-box 2 (SOX2) as reliable markers of multipotency of NSCs (Ellis et al., 2004; Wiese et al., 2004).

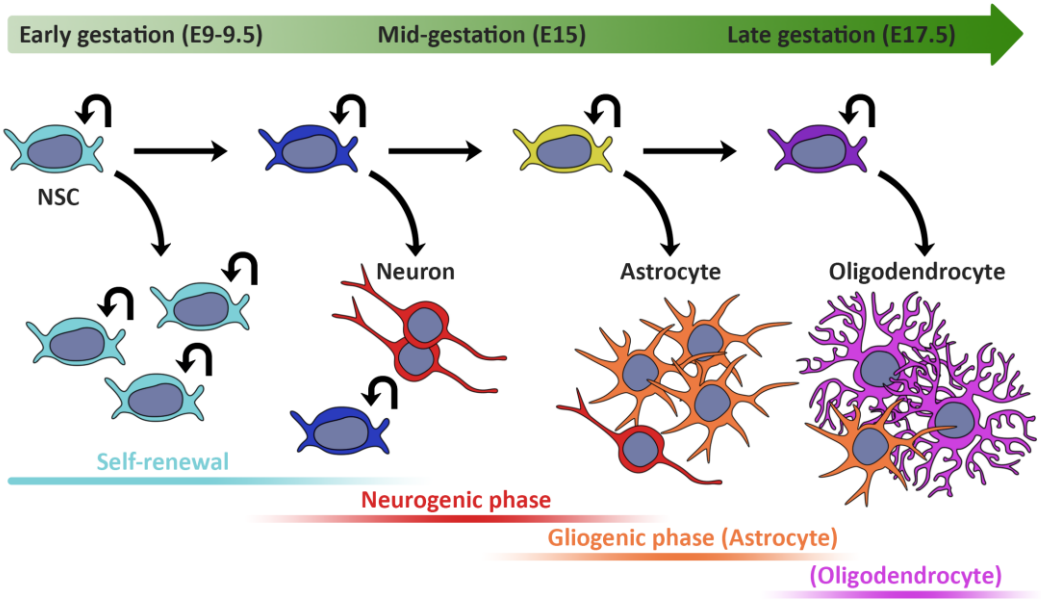

Figure 1.4: Embryonic neurogenesis. During early gestation (E9-E9.5 in rodents) stem cells (light blue) self-renew and expand their pool to generate progenitors of the different neural cell lineages. During mid-gestation (E15 in rodents) the neural stem cells produce neuronal progenitors (dark blue), which continue asymmetric division to populate the CNS with neurons (red cells). The gliogenic phase begins when glia progenitors (yellow) generate mainly astrocytes (orange). During the late gestation (E17.5 in

rodents), the oligodendrocytic progenitors (dark purple) give rise to mainly oligodendrocytes (light purple) and some astrocytes.

1.2.2 Postnatal hippocampal neurogenesis

The postnatal period of the rodent is markedly different from that in humans in terms of cellular and system development. For instance, oligodendrocyte maturation (Craig et al., 2003), immune system development (Holsapple et al., 2003) and blood-brain barrier development (BBB; (Engelhardt, 2003)) take place at postnatal day (P) 1-P3 in rodents, while for humans these processes take place prenatally (Daneman et al., 2010; Dean et al., 2011). During later postnatal development, between P7 and P10, rodents exhibit their highest brain growth spurt (Bockhorst et al., 2008), highest level of generating glial cells (Catalani et al., 2002; Kriegstein and Alvarez-Buylla, 2009) and growth of axonal and dendritic density (Baloch et al., 2009; Bockhorst et al., 2008). In this period the development of the rodent immune system is consolidated (Holsapple et al., 2003). In the final stage of postnatal development, just before the juvenile period commences at about P20 - P21, the synaptic density in the rodent brain peaks (Micheva and Beaulieu, 1996), being twice as high as the synaptic density observed in adults. Additionally, changes in neurotransmitters and their receptors are also observed (Micheva and Beaulieu, 1996; Romijn et al., 1991).

Not much is known about the rate of early postnatal neurogenesis in the rodents. However, it has been shown that within the hippocampus there is a rapid and robust increase of glial fibrillary acidic protein (GFAP) -expressing cells, possibly astrocytes, reaching peak between P11 and P16 (Catalani et al., 2002). This growth coincides with

the rodent critical period of synaptogenesis in the molecular layer of the DG. Up until P4 the synaptic density represents less than 1% of what is observed in the adult brain. At P14 peak levels of synaptogenesis occurs which then levels off at P25 to that consistently observed in the adult hippocampus (Crain et al., 1973). This concurrence of events is interesting in the light of recent findings that astrocytes can modulate neuronal activity in the young rodent brain in response to glutamatergic transmission through the metabotropic glutamate receptor 5 (mGluR5), the expression of which however is significantly decreased after P14 in astrocytes of the rodent hippocampus (Sun et al., 2013b). This suggests a possible mechanism driven by asotrcyte-mGluR5-mediated regulation of synaptogenesis and also of fundamental differences in the neuronal-astrocyte signalling between the immature and adult brains (Semple et al., 2013; Sun et al., 2013b).

While most of the granule cells of the rodent hippocampus are generated up until P10 (Altman and Bayer, 1990; Piatti et al., 2006), RGCs remain abundant in the developing brain until P14 (Malatesta et al., 2000) when they start differentiating into NPCs. This is followed by their transformation to neuroblasts and finally to mature excitatory granule cells that integrate in the circuitry by P21 (Kriegstein and Alvarez-Buylla, 2009). These changes have been confirmed in nestin-eGFP (green fluorescent protein) transgenic mice, where a comprehensive analysis of NPCs in mice aged from P7 and P28 revealed that, not only did the number of NPCs decrease over this developmental period, but also that the genetic profile of the NPCs from the two ages was markedly different and this thus suggests early adulthood senescence (Gilley et al., 2011).

Furthermore, such results were replicated in a recent study employing single-cell RNA (ribonucleic acid) sequencing and *in situ* hybridization. The data show that the process of neurogenesis in the DG undergoes a transformation from the embryonic to the postnatal period represented by a shift in RGCs around P14 from an embryonic to an adult state. This process was coupled with an increase in NSC differentiation towards neuroblasts rather than glia (Hochgerner et al., 2018). While the authors argue that NPCs and neuroblasts sustain very similar properties throughout later stages of life, they have identified a distinct phenotype of the adolescent and adult hippocampal granule cells of the rodent, which will be discussed in detail in the following sections.

1.2.3 Adolescent hippocampal neurogenesis

The levels of neurogenesis in the adolescent rodent hippocampus are much higher compared to adults as illustrated by a mouse study comparing the number of BrdU+ and DCX+ cells between adolescent (P30) and adult mice (P120). There was a 4-fold decrease in the number of proliferating (BrdU+) NPCs and the number of immature neurons (DCX+) in the hippocampi of adult mice, when compared to the proliferative and neurogenic levels present in the hippocampi of the adolescent animals (He and Crews, 2007). This finding was consistent across neurogenic areas (both SGZ and SVZ were examined) and across the wildtype and transgenic mouse models that were employed in the study (the transgenic mouse models used were two: overexpression of either IGF-1 or IGFBP-1 (He and Crews, 2007)). Thus, it was suggested that adolescence is associated with high levels of neurogenesis, while the transition from adolescence to adulthood correlates with a dramatic reduction in neurogenesis (He and Crews, 2007). The mechanisms by or purpose for which this occurs are poorly

understood and only a limited number of studies have focused on investigating the process of hippocampal neurogenesis during adolescence. Interestingly, it has been shown that the process and time course of apoptosis of newborn granule cells in the adolescent DG closely reflects that of the adult DG, albeit to an exaggerated degree (Curlik et al., 2014). Juvenile rats (P21-P23) were administered intraperitoneal (i.p.) injection of BrdU and the number of cells, which incorporated BrdU was analysed one- and three-weeks post injection. It was found that more than 7 000 proliferating cells retained BrdU expression within a week. However, most of them were no longer detected 3 weeks after the injection, indicating a similar rate of apoptosis as in the adult hippocampus (Curlik et al., 2014; Epp et al., 2007). Survival of the proliferating cells was significantly enhanced for animals that underwent trace eye blink conditioning, which is considered a task that requires effortful learning (Curlik et al., 2014). Additionally, findings from our lab have illuminated an age-specific effect of voluntary exercise such that, adolescent-initiated running led to an increased expression of a wide array of plasticity- and neurogenesis-related genes in the hippocampi of male Sprague Dawley (SD) rats. Among the upregulated genes were *bdnf* (brain-derived neurotrophic factor), *tlx* (the human homologue of the Drosophila *tailless* gene) and *dcx*, the functions of which will be discussed in subsequent sections and the pre- and post- synaptic regulating genes *synaptophysin* and *psd-95* (postsynaptic density protein 95; (O'Leary et al., 2018)).

Much research has concentrated on investigating the effects of alcohol exposure during adolescence on brain development and cognitive behaviour (reviewed by

(Crews et al., 2016)). Interestingly, it has been consistently shown that hippocampal neurogenesis is significantly reduced in adolescent and adult rodent models of binge drinking. However, while neurogenesis recovers to normal levels in adults after a period of abstinence, when the alcohol exposure took place during adolescence rather than adulthood the deficit persists until late adulthood (Crews et al., 2007). Such a long-lasting reduction in hippocampal neurogenesis was also observed after administration of alcohol to adolescent rhesus monkeys (Taffe et al., 2010). Furthermore, binge ethanol exposure during adolescence has been shown to directly influence NSCs and NPCs but not neuroblasts, by reducing proliferation and increasing apoptosis in cells of both the dDG and vDG of rodents (rats; (Vetreno and Crews, 2015)) and non-human primates (rhesus monkeys; (Taffe et al., 2010)). This was also coupled with impaired cognitive performance on memory tests (Taffe et al., 2010; Vetreno and Crews, 2015). Moreover, during the abstinence period in adolescent rodents, neuroblasts were ectopic, and were found in the molecular layer of the DG rather than within the SGZ (McClain et al., 2014). These studies, not only highlight the sensitivity of the adolescent brain to alcohol neurotoxicity, but also emphasize that decreased hippocampal neurogenesis during adolescence may be an important risk factor for the development of neuropathology and/or cognitive deterioration (Crews et al., 2016).

1.2.4 Adult hippocampal neurogenesis

Adult neurogenesis encompasses several consecutive phases of development, which are preserved in the adult brain and result in the production of new neurons (Goncalves et al., 2016). However, it is noteworthy that neurogenesis occurs in the DG alone,

which based on past terminology is not even part of the hippocampus proper but rather part of the hippocampal formation, thus what adult hippocampal neurogenesis really refers to is neurogenesis in the adult DG (Kempermann et al., 2015). The first piece of evidence to show the existence of adult hippocampal neurogenesis emanated half a century ago. However, the first direct data set illustrating functional integration of newly born neurons in the adult CNS followed 20 years later from a study of songbirds (Paton and Nottebohm, 1984). After another decade a crucial advance occurred – the introduction of BrdU to the study of adult neurogenesis (Kuhn et al., 1996). BrdU is a synthetic nucleotide analogue to thymidine which incorporates into proliferating cells, and thus allows for investigating their cell fate at later timepoints (Russo et al., 1984). This along with the development of retroviral methods for tracing cells from birth and marking specific genetic markers (van Praag et al., 2002), in combination with immunohistochemistry (IHC), confocal imaging and electrophysiology allowed for the field of neurogenesis to take off. The rapid progress during the past decades along with the discovery of adult hippocampal neurogenesis in the human brain (Eriksson et al., 1998) has brought about soaring interest on the topic among the public and scientific community.

The cells comprising the adult SGZ are the NPCs along with their progeny, immature neurons, glial and endothelial cells, immune cells, microglia and macrophages, and extracellular matrix (Kempermann et al., 2015). The only neuronal type known to date to be produced through adult hippocampal neurogenesis is the granule cell (Kempermann et al., 2015). It has been proposed that γ-aminobutyric acid (GABA)-

ergic inhibitory neurons or basket cells are also generated in the adult rat hippocampus (Liu et al., 2003), however, the evidence has not been confirmed. The excitatory granule neurons, whose input areas and the areas they project to were described previously (see Figure 1.2), exhibit sparse action potentials, are modulated by interneurons in the DG and hilus, and arise from the precursor cells of the SGZ, also known as the neurogenic niche of the hippocampus (Kempermann et al., 2015). The term "niche" refers to an anatomical area, wherein the stem cells reside owing to the special microenvironment created by the niche. In the case of the SGZ, it is permissive for neurogenesis and neuronal development to occur (Ming and Song, 2011). The term was first coined in the study of transplantation of hematopoietic progenitors (Schofield, 1978). It has been suggested that such niches are surrounded by a common basal membrane (Mercier et al., 2002). Since the vasculature is a very important factor for the maintenance and function of the hippocampal neurogenic niche, the term "vascular niche" has also been proposed (Palmer et al., 2000). The NSCs interact closely with the endothelial cells in a multifaceted fashion (Wurmser et al., 2004) and also extend processes, called "end feet", connecting to the vasculature of the SGZ (Filippov et al., 2003). Additionally, vascular endothelial growth factor (VEGF) has been shown to be a robust modulator of adult hippocampal neurogenesis (Jin et al., 2002; Schanzer et al., 2004). Along with the vasculature, the neurogenic permissiveness of the SGZ is brought about due to the distinctive environment of various circulating growth factors, unique extracellular matrix and cell-to-cell contacts, with astrocytes being the most prominent supporting cells for the neurogenic process (Kempermann et al., 2015). For instance, astrocytes in the SGZ have been found in very close proximity to proliferating cells and newborn neurons throughout

their developing phase (Plumpe et al., 2006; Shapiro et al., 2005), while astrocytes in hippocampal primary cultures along with astrocyte-secreted molecules have been shown to prompt the neurogenic process (Barkho et al., 2006; Song et al., 2002). Furthermore, the neurogenic niche of the hippocampus is innervated by neurons from a multitude of brain areas which employ different neurotransmitter systems (which will be discussed below) as well as by commissural tracts from its contralateral side (Kempermann et al., 2015).

The process of adult neurogenesis consists of four stages (nomenclature adopted from (Kempermann et al., 2015)): a precursor stage, an early survival stage, a postmitotic maturation stage and a late survival stage. The stages can be further divided into six events or transformations that the cells undergo based on evaluating their morphology and protein expression (see Figure 1.5; (Kempermann et al., 2004; Kempermann et al., 2015; Steiner et al., 2006)). Firstly, the precursor stage (also known as expansion stage) when NSCs go through three continuous progenitor phases characterized by elevated proliferation levels, is followed by the early survival stage when NPCs exit the cell cycle and the number of newborn neurons significantly decreases due to elimination. Next, the postmitotic stage is characterized by dendritic and axonal outgrowth, synaptogenesis and the establishment of connections. Finally, the late survival stage, marks the integration of new granule cells into the existing circuitry after fine-tuning of their connectivity (reviewed by (Kempermann et al., 2004; Kempermann et al., 2015)). The duration from the first to final stage of neurogenesis is thought to last about 7 weeks, while the process from the birth of a new neuron

(NSC asymmetric division) to its complete integration in circuitry has been suggested to last 4 weeks (Goncalves et al., 2016; Ming and Song, 2011).

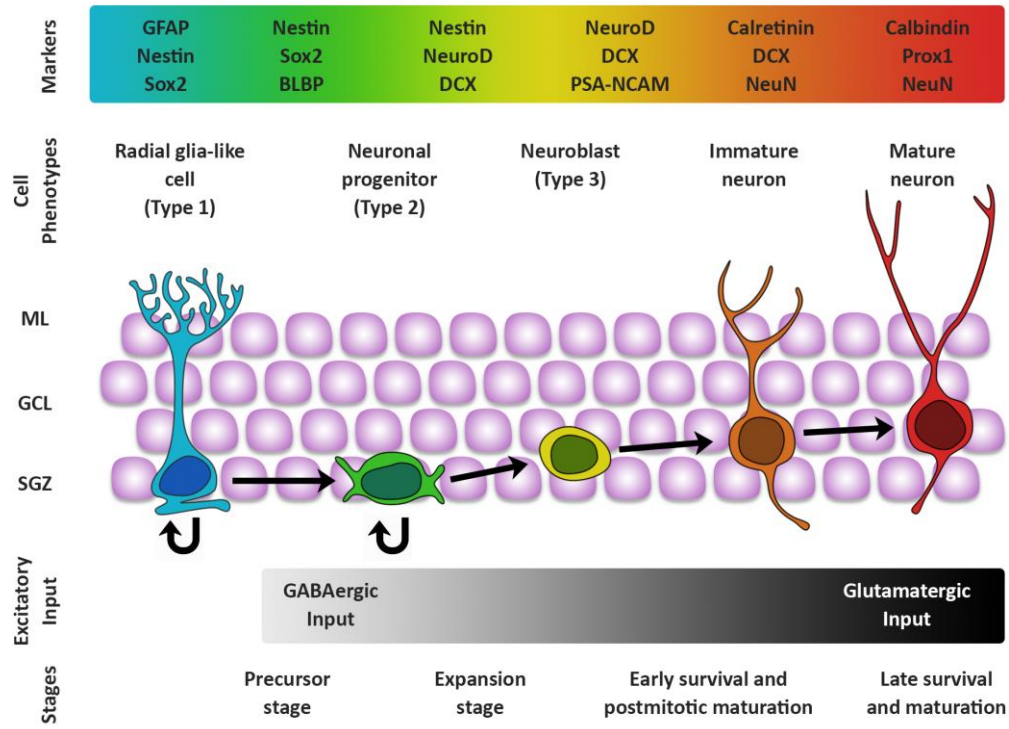

Figure 1.5: Stages of hippocampal neurogenesis. Depiction of the stages of the neurogenic process in the hippocampus. The radial glia-like stem cells (Type 1; blue) maintain their pool through self-renewal and give rise to progenitor cells expressing similar markers but displaying different morphology (Type 2 (A&B); green), which undergo rapid proliferation and begin to express markers specific to the neuronal fate of their progeny. Type 2 cells generate neuroblasts (Type 3; yellow). The neuroblasts enter the early survival stage (orange cells) and extend processes towards the molecular layer. During the late survival stage, only newborn neurons that have formed functional connections and have matured morphologically (red cells) remain from the thousands of neuroblasts generated. Granule neuron somata are represented in purple. The colour-coded bar on top illustrates the gradual transition in marker expression as the cells progress through the different stages of the neurogenic process. The grey-gradient-scale bar on the bottom represents the switch of newborn neurons from GABA to glutamatergic input. ML: molecular layer; GCL: granule cell layer; SGZ: subgranular zone.

1.2.4.1 The precursor stage

During the precursor stage, several different types of NPCs can be observed within the SGZ. They exhibit distinct morphological features but do not represent separate cell populations, rather they emulate different phases or events from the continuum of adult neurogenesis (Kempermann et al., 2015). The cells giving rise to adult hippocampal

neurogenesis present morphologically and antigenically as radial glia (Type 1), yet some of the markers of embryonic NSCs are absent from the adult hippocampal NSCs, with their soma located to the SGZ and a single process travelling to and branching out within the molecular layer of the DG (Zhang and Jiao, 2015). The origin of Type 1 cells was first demonstrated through pharmacological elimination of mitosis within the DG, followed by examination of the phenotype of the first proliferative cells to re-emerge. The cells presented an astrocyte-like radial morphology (Seri et al., 2001). Subsequently, the same group of researchers established that the cells giving birth to new neurons in the adult SGZ expressed GFAP and nestin (Seri et al., 2004), both of which are well-known markers of astrocytes (Szymas et al., 1986) and embryonic and adult NSCs, respectively (Gilyarov, 2008; Lendahl et al., 1990; Park et al., 2010a). In a further attempt to characterize hippocampal Type 1 cells Filippov et al. showed that the nestin+ radial-glia-like cells exhibited an electrophysiological phenotype similar to that of astrocytes (Filippov et al., 2003), namely a passive current and a reversal potential close to the potassium equilibrium potential (Steinhauser et al., 1992). Additionally, these cells lacked the expression of the astrocyte-specific marker calcium binding protein β, also known as S100β (Filippov et al., 2003). Lastly, the brain lipid binding protein (BLBP) – a well-established radial-glia cell marker (Brunne et al., 2010; Pinto and Gotz, 2007) along with the transcription factor SOX2 – a robust marker of embryonic and adult NSCs (Avilion et al., 2003; Rizzino, 2009; Suh et al., 2007) were also found to be expressed by Type 1 cells ((Steiner et al., 2006); See Figure 1.5). It is worth noting that the "true stemness" of Type 1 cells or their "unlimited" potential to self-renew has been contested. *In vitro* studies of primary subependymal and hippocampal cultures have found that only cells isolated from the

lateral walls of the ventricles exhibited long-term self-renewal capacity and multipotency, while cells isolated from the DG displayed neuro-progenitor rather than a NSC phenotype (Bull and Bartlett, 2005; Seaberg and van der Kooy, 2002). Several years later, however, the Kempermann lab optimized a method of isolation and propagation of NPCs from the adult murine DG, allowing for consistent procurement of hippocampal progenitors which exhibited stem-cell-like properties such as continual self-renewal capacity (Babu et al., 2007). Nonetheless, findings from *in vivo* studies have not aided in resolving the issue of "stemness" in hippocampal NPCs (Kempermann, 2011). While Type 1 cells have been proposed to only undergo asymmetric division (Encinas et al., 2011), the opposite has been also shown, namely symmetric division and thus unlimited self-renewal of Type 1 cells (Bonaguidi et al., 2011). However, the lineage analyses of the cells employed by both groups were different – the former study adopted an *in vivo* genetic fate-mapping strategy in combination with readout of Shh activity (Encinas et al., 2011), while the latter study employed an *in vivo* clonal lineage tracing (Bonaguidi et al., 2011). These results reflect the heterogeneity of NSCs and NPCs in the neurogenic niche and the importance of establishing common concepts of the neurogenic stages and markers that can be used to delineate them (Kempermann et al., 2015). Furthermore, it has been proposed that Type 1 cells are quiescent stem cells that enter the cell cycle upon activation (Lugert et al., 2010). Various factors which transcriptionally regulate or repress inhibitors of the NSC signalling pathways control cell cycle exit and entry of Type 1 cells (Goncalves et al., 2016). These transcription factors will be discussed in more detail in subsequent section. To sum up, the population of NPCs within the SGZ of the hippocampus is a very heterogeneous one with the quiescent Type 1 radial glia-

like NSCs first in the chain of different types of progenitors that propagate the expansion of the NSC pool. It has been debated whether this population of cells exhibit stemness, specifically the capacity to self-renew interminably (Kempermann, 2011).

1.2.4.2 The expansion stage

The resulting Type 2 cells display a different morphology and can be further divided into two subcategories – Type 2a and Type 2b. Type 2 cells are the intermediate progenitors of adult born granule neurons with transient amplifying characteristics (Kempermann et al., 2015). Their proliferation constitutes the expansion phase of neurogenesis, when the number of NPCs increases dramatically. The main difference between Type 1 and Type 2a cells is in morphology, while the main difference between Type 2a cells and Type 2b cells is in marker expression. Type 1 and Type 2a cells both express nestin, BLBP and SOX2. While Type 1 cells present the radial-glia-like morphology and express GFAP, Type 2a lack the radial processes and may or may not express GFAP (Steiner et al., 2006). Additionally, patch-clamp electrophysiological experiments showed that the two populations of cells (radial glia-like stem cells of Type 1 and the transiently amplifying progenitors of Type 2) also displayed different input resistance values (See Figure 1.5; (Fukuda et al., 2003)). Type 2 progenitors display highly proliferative activity, which has been shown to be positively and negatively modulated by exposure to voluntary wheel running (Kronenberg et al., 2003) and stress (reviewed by (Kino, 2015)), respectively. Additionally, the proliferative activity of Type 2 cells has been shown to intensify as a result of antidepressant intake through serotonin-mediated mechanisms (Encinas et al., 2006). Furthermore, Type 2 cells receive excitatory GABAergic input which has

been suggested as key regulator of their proliferation and differentiation (Tozuka et al., 2005; Wang et al., 2005).

Type 2a and Type 2b are considered to be the link as well as the delineating mark between glial and neuronal fate in adult hippocampal neurogenesis. Specifically, Type 2a cells have the potency to adopt either, while Type 2b cells are restricted to a neuronal lineage (Steiner et al., 2006). This is also reflected by their marker expression; Type 2a express mostly glial markers (BLBP, SOX2, nestin and in some cases GFAP) and Type 2b cells express the pro-neuronal transcription factors neurogenic differentiation 1 (NeuroD1) and prospero homeobox protein 1 (PROX1) as well as the polysialylated neural cell adhesion molecule (PSA-NCAM) and the neuronal migration protein DCX (reviewed by (Zhang and Jiao, 2015)). The transition from Type 2a to Type 2b cells has been suggested to occur in a gradient-like manner such that the existence of a mixed phenotype of Type 2ab cells has also been proposed (Zhang and Jiao, 2015). This transition is driven by a delicately orchestrated repression and activation of transcription factors which regulate the fate of the cells in the neurogenic niche. This cascade of events (activation/repression of transcription factors) is conducted through epigenetic mechanisms, neurotransmitter signalling, as well as though growth factor and morphogen release within the neurogenic niche (Faigle and Song, 2013). An example of the gradient expression of different transcription factors on Type 2a and 2b cells is illustrated below, however, the molecular regulation of this expression is discussed in subsequent section of this chapter. For instance, SOX2 and TLX are strongly expressed in Type 1 and Type 2a

cells, and their downregulation co-occurs with the upregulation of the T-box transcription factor Eomes (Tbr2), PROX1 and NeuroD1, with the latter marking the transition of the progenitor to a Type 2b NPC phenotype (Faigle and Song, 2013). Studies of constitutive and conditional knockout mouse models have shown that adult neurogenesis cannot persist in the absence of these transcription factors (Brulet et al., 2017; Christie et al., 2006; Hodge et al., 2008; Hodge et al., 2012; Karalay et al., 2011; Young et al., 2002). Type 2b cells also mark the start of DCX and PSA-NCAM expression, markers which are maintained from the expansion and proliferation stage of the neurogenic process through the exit of cell cycle, early maturation and survival stage of the newborn granule cells (See Figure 1.5 (Couillard-Despres et al., 2005; Zhang and Jiao, 2015)). Despite it being dispensable for neuronal development during adult hippocampal neurogenesis (Merz and Lie, 2013), DCX is conventionally used as a surrogate marker for adult neurogenesis (Kempermann et al., 2015).

1.2.4.3 The early survival and postmitotic maturation stage

Type 3 cells, also known as slowly proliferating neuroblasts, further contribute to the expansion phase but significantly less so than Type 2 cells (Dokter and von Bohlen und Halbach, 2012). Nevertheless, under pathophysiological conditions such as experimentally induced seizures, the proliferative activity of Type 3 cells is dramatically increased (Jessberger et al., 2005). Type 3 cells cease to express nestin, but they retain DCX, PSA-NCAM, NeuroD1 and PROX1 expression and also continue to receive excitatory GABAergic input (See Figure 1.5 (Kempermann et al., 2015)). Type 3 cells initiate a short distance migration from the SGZ to the GCL where they exit the cell cycle and undergo a morphological developmental transition, such

that they extend processes in a vertical direction. This marks the transition to the early postmitotic maturation phase of the neurogenic process (Dokter and von Bohlen und Halbach, 2012; Kempermann et al., 2015).

Promptly upon exit of the cell-cycle, Type 3 cells display expression of the post-mitotic neuronal nuclei (NeuN) marker and the Ca^{2+} -binding protein calretinin, which is only transiently expressed during the early, but not late survival phase of the neurogenic process (Brandt et al., 2003; Zhang and Jiao, 2015). The colocalization of BrdU and NeuN is a useful tool for examining survival rate of newborn neurons, by varying the time points of investigation post BrdU administration (Zhao et al., 2013b). It has been shown that the number of BrdU+NeuN+ cells is highest during the early post-mitotic phase after BrdU administration, and dramatically decreases over the next days into the late survival phase due to an apoptotic elimination process (Biebl et al., 2000; Kempermann et al., 2015). The mechanisms regulating the survival versus apoptosis of a neuroblast within the GCL remain elusive, but various candidates such as neurotransmitters (Tashiro et al., 2006), growth factors (Pfisterer and Khodosevich, 2017), B-cell lymphoma 2 (Bcl-2; (Kuhn et al., 2005)), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB; (Imielski et al., 2012)) and tumour protein p63 (p63; (Cancino et al., 2013)) are proposed to be involved. The neuroblasts that survive the initial apoptotic elimination proceed with dendritic development and axon extension towards the CA3 region, where synapses with target cells are formed (Sun et al., 2013a). The axons of adult-born granule cells become part of the mossy fibre tract and significantly contribute to its plasticity and size, both of which have been

correlated with the rate of adult neurogenesis (Romer et al., 2011). During this stage, glutamatergic synaptic input begins to innervate the cells and the GABA switch from being excitatory to inhibitory occurs once there is sufficient glutamatergic input or once the granule cells adopt glutamatergic phenotype (Tozuka et al., 2005). At this stage the apoptotic elimination is significantly reduced, with a small decrease in newborn neurons two weeks post mitosis (Kempermann et al., 2015). Retroviral labelling of adult-born neurons, which allows fluorescent tagging of neurons undergoing mitosis at the time of virus administration, revealed that axons extend to CA3 around 10 days after the cell has been labelled, while dendritic spines only begin to emerge 17 days post mitosis (Zhao et al., 2006).

1.2.4.4 The late survival and maturation stage

The final stage of adult neurogenesis encompasses the late maturation phase of the newborn neurons. This phase takes place around 3-4 weeks post mitosis when the cells cease to express the immature neuronal markers DCX and PSA-NCAM, while they retain the expression of NeuN, PROX1 and NeuroD1 and switch their Ca^{2+} -binding protein from calretinin to calbindin (See Figure 1.5 (Brandt et al., 2003; Zhang and Jiao, 2015)). Though at this phase cells appear to be morphologically mature and fully integrated into the existing circuitry, they still exhibit different electrophysiological properties from mature neurons, namely that the newborn granule cells are a lot more excitable with a significantly lower threshold for long-term potentiation (LTP) induction than their mature counterparts (Schmidt-Hieber et al., 2004; van Praag et al., 2002). This enhanced synaptic plasticity is normalized or inhibited by the local interneurons approximately 1-1.5 months post mitosis, when the late maturation phase

is assumed to conclude as the adult-born granule cell becomes indistinguishable from a granule neuron that has been there since birth (Ge et al., 2007; Marin-Burgin et al., 2012). In subsequent sections, the function and regulation of this fascinating multistep process will be discussed in detail.

Throughout embryonic, postnatal and adult neurogenesis the four stages of neuronal development or neurogenesis along with the six milestones remain conserved. Moreover, excitatory GABA signalling followed by inhibitory GABA signalling, takes place across all developmental stages (Ge et al., 2008; Ming and Song, 2011). However, the adult neurogenic process diverges from embryonic and postnatal neuronal development mainly due to the duration of neuronal maturation – it is much faster pre- and postnatally rather than in adulthood (Overstreet-Wadiche et al., 2006; Zhao et al., 2006).

1.2.5 Hippocampal neurogenesis during ageing

Hippocampal neurogenesis is presumed to persist throughout the life span, however, a decline in neurogenesis has been recognized to occur with age across species. In fact Altman and Das (1965) in their pioneering paper commented on the decrease of cell birth within months after birth (Altman and Das, 1965a; Kempermann, 2015; Klempin and Kempermann, 2007). The first report to quantify age-related changes in adult hippocampal neurogenesis came from a study of 12- to 21-month old rats where the authors showed, through BrdU labelling and IHC analysis immediately after, or 4 to 6 weeks after BrdU administration, that a decrease in mitotic activity of NPCs in the

SGZ occurred and was associated with a net decrease in neurogenesis (Kuhn et al., 1996). Furthermore, Kempermann and colleagues showed a similar age-related decrease in hippocampal neurogenesis in 8- to 20-month old mice, and the decrease in neuronal survival could be somewhat ameliorated by enriched housing conditions (Kempermann et al., 1998). The age-associated decrease in hippocampal neurogenesis has also been shown in tree shrews. This study also demonstrated that older animals were more susceptible to a stress-induced decline in NPC proliferation than their younger counterparts (Simon et al., 2005). Interestingly, despite illustrating a net decrease in hippocampal neurogenesis in wild-living aged squirrels and chipmunks, it was shown that there was a species difference in terms of the age-related decrease observed. Specifically, the number of proliferating NPCs was decreased in the DG of squirrels, while the number of immature adult born neurons was diminished in the DG of chipmunks (Barker et al., 2005). This finding is particularly interesting, in light of highlighting the complexity of studying the neurogenic process in non-captive populations, since the squirrels were relying on neurogenesis-dependant strategies (spatial memory) to locate their hidden food stores, while the chipmunks had much less developed spatial memory and relied on single place for storing their food (Barker et al., 2005). Aside from rodents, it has been shown using BrdU incorporation, that hippocampal neurogenesis persists in non-human primates, namely the Macaque monkeys, until they are 23 years old (the human equivalent of old age). However, the rate of neurogenesis occurred at significantly lower levels than during adolescence and adulthood (Gould et al., 1999b). Similarly to what has been reported on adult human hippocampal neurogenesis, this existence of an age-related decline in neurogenesis remains controversial (Snyder, 2018). Intriguingly, the researchers who propose the

occurrence of neurogenesis in the adult human hippocampus, have not found a decline of NPC proliferation or of neurogenesis with age (Boldrini et al., 2018; Eriksson et al., 1998), despite a reported age-associated decline of the quiescent progenitors pool (Boldrini et al., 2018).

The mechanisms underlying the age-related decline in hippocampal neurogenesis remain poorly understood. It has been proposed that within the senescent brain the neurogenic niche may be deprived of the extrinsic signals regulating the neurogenic process or that the aged NPCs are less responsive to normal signalling within the niche, or both (Kempermann, 2015). The evidence accumulated thus far points to changes in the properties of the neurogenic niche with age, rather than changes in the phenotype of the NS/PCs themselves. For instance, it has been reported that the numbers of NSCs and NPCs as well as the proportion of astrocytes to neurons in the hippocampus of young and aged rats remained the same, however, there was a decrease in the number of cells actively undergoing mitosis in the aged animals (Hattiangady and Shetty, 2008). The authors speculated that this was due to changes in the milieu of the neurogenic niche based on their earlier observations that important regulators of neurogenesis such as BDNF and CREB (cyclic adenosine monophosphate (cAMP)-response element binding protein) decreased dramatically in the DG of middle-aged and aged rats (Hattiangady et al., 2005). Additionally, it was shown that the gradual loss of hippocampal neurogenesis in aged mice was associated with downregulation of the mitotic factor survivin in a Wnt-dependent signalling manner (Miranda et al., 2012). This finding was corroborated with the observation that the Wnt antagonist

Dickkopf-1 increased with age, while mice deficient in Dickkopf-1 not only exhibited enhanced hippocampal neurogenesis during aging, but also performed better at neurogenesis-dependent tasks, involving spatial working memory, than age-matched controls whose Dickkopf-1 expression was not modulated (Seib et al., 2013). Growth factors such as epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1), which are important regulators of adult neurogenesis (to be discussed in detail in subsequent section) have also been shown to play role in the age-related decline of hippocampal neurogenesis in the rodent (reviewed by (Kempermann, 2015)). For instance, a combination of BrdU and DCX labelling of cells in both neurogenic niches of the mouse brain (the SVZ and the SGZ) showed that by 20 months of age there was a 90% decrease in newborn neurons in the hippocampus and a 50% decrease in the SVZ compared to the brains of 3-month old mice. Interestingly, intracerebroventricular (i.c.v.) administration of basic fibroblast growth factor (FGF-2) and EGF resulted in not only an alleviation of the decrease in neurogenesis, but also an enhancement of the number of adult born neurons in the aged hippocampus, illustrating that the aged brain is still susceptible to the influence of exogenous growth factors (Jin et al., 2003). What is more, the levels of FGF-2 as well as IGF-1 and VEGF, were found to dramatically decline in the hippocampi of aged rats. Additionally, the authors showed that the FGF-2 decline was as a result of an age-related deterioration of FGF-2 synthesis by astrocytes, leading to a reduced number of GFAP+FGF2+ radial-glia-like cells in the DG of aged rats (Shetty et al., 2005). It was also independently demonstrated that the hippocampus is one of the regions of the rat brain with the highest and most robust expression of the FGF-2 receptor FGFR2, specifically on astrocytes, and that the expression of this protein decreased

significantly with age (Chadashvili and Peterson, 2006). Infusion of IGF-1 through i.c.v. ameliorated the decrease of hippocampal neurogenesis in aged rats (Lichtenwalner et al., 2001), while in a model of long-lived mice (the Ames dwarf mice) enhanced hippocampal neurogenesis coupled with increased levels of IGF-1 were observed during aging (Sun et al., 2005). Another prominent perpetrator of the age-related decline in hippocampal neurogenesis has been proposed to be the family of glucocorticoid hormones and receptors, the release and circulation of which coincidentally increase with age (Cameron and McKay, 1999). Last but not least, another piece of evidence supporting the impairment in neurogenic niche properties over time stems from a study where aged mice were infused with vascular and neurogenic factors of young mice which resulted in rejuvenated neurogenic niche and restored hippocampal neurogenic levels (Katsimpardi et al., 2014). Furthermore, despite the lack of alterations in properties of hippocampal Type 1 and Type 2 cells with age, a group of researchers illustrated through IHC labelling of cells with BrdU and NeuN a possible delayed maturation of adult born neurons in the aged DG (Rao et al., 2005). Taken together, these findings highlight the complex interplay of different factors within the neurogenic niche that may be affected by mechanisms driven through the ageing process and thereby ultimately affect the number of immature neurons produced in the aged brain. The functional implications of these neurons are discussed below.

1.3 Function of hippocampal neurogenesis across the life span

1.3.1 Function of hippocampal neurogenesis during embryonic and postnatal development

The function of neurogenesis during embryonic development is to populate the various regions of the CNS with different types of neurons derived from NSCs of the neural tube (Kandel, 2000). The hippocampal formation is largely developed by E20 in rodents (Bayer, 1980), and by 20 weeks of gestation in humans (Gomez and Edgin, 2016). However, volumetric development persists to P21 in rodents and to 2 years of age in humans (Ainge and Langston, 2012; Hevner, 2016). The DG is the last hippocampal structure to appear at E16 in rats with the GCL formed at E21. It undergoes a 50% daily volumetric increase from P1 to P7, and 21% from P7 to P21, when growth and neurogenesis levels level off (Bayer, 1980). The heightened level of neurogenesis during early postnatal days in rodents and the first years in humans has been linked to the phenomenon of infantile amnesia, or the absence of memory for events that have taken place within the first 2-3 years of life as well as the vague recollections of occurrences from 3 to 7 years of age (Josselyn and Frankland, 2012). The existence of infantile amnesia has been demonstrated across a wide range of tasks such as conditioned suppression (Campbell and Campbell, 1962), passive (Schulenburg et al., 1971) and active (Klein and Spear, 1969) avoidance (Feigley and Spear, 1970), appetitive discrimination (Campbell et al., 1968), contextual fear conditioning (Rudy and Morledge, 1994; Weber et al., 2006), spatial navigation in the

MWM (Brown and Kraemer, 1997) and novel object recognition (NOR; (Anderson et al., 2004)). Given that each of these tasks has rather distinct characteristics in terms of the stimuli employed and performance demands, in addition to the consistent results illustrating that memory persistence is proportional to age at time of acquisition, it has been concluded that infantile amnesia occurs during early postnatal development across species (Josselyn and Frankland, 2012). Direct evidence linking neurogenesis and infantile amnesia came from experiments where neurogenesis was manipulated (inhibited or enhanced) during different points of the life span. The results illustrated that increasing neurogenesis promoted forgetting in adult mice, while reducing neurogenesis facilitated memory persistence in infant mice (Akers et al., 2014). To sum up, the function of early life postnatal hippocampal neurogenesis appears to be related to weakening existing memories and information storage in favour of strengthening the ability to learn new things and to acquire new information through rapid continuous generation of large number of new granule cells (Akers et al., 2014; Josselyn and Frankland, 2012). The adaptive value of this function is considered to be in the rapid clearance of old information that may not be useful, in order to facilitate increased capacity and reduced interference between memories.

1.3.2 Function of hippocampal neurogenesis during adolescence

A classic approach in understanding the function of hippocampal neurogenesis is by employing different ablation techniques. Several studies have investigated whether inhibiting hippocampal neurogenesis during adolescence results in similar impairments as observed when the process was inhibited in adulthood. For instance, cranial irradiation of the rat hippocampal region during pre-adolescence resulted in

dramatic apoptosis and impaired production and release of growth factors in the hippocampus, while the same procedure performed in adulthood resulted in sustained release of pro-inflammatory cytokines in the hippocampus (Blomstrand et al., 2014). A chronic social defeat paradigm induced a transient reduction in the number of proliferating NPCs in the hippocampi of adolescent but not adult male mice suggesting a resilience by adolescent mice to impairments induced by the stress. This phenomenon could not be accounted for by the damage to emotional processing and sociability caused by the inhibition of hippocampal neurogenesis since both adolescents and adults exhibited normal performance on depression-related behavioural tests as well as a regular corticosterone response after acute exposure to stress (Kirshenbaum et al., 2014). Interestingly, using the same method of transient ablation of hippocampal neurogenesis, another group of researchers found the same outcome of memory and anxiety-related behaviours for both adolescent and adult female mice. However, impaired female-female social interaction resulted when neurogenesis was inhibited during adolescence but not adulthood, reflected by a complete social aversion towards conspecifics, as well as impaired pup retrieval (Wei et al., 2011). Furthermore, in a study comparing the effect of the antidepressant fluoxetine administered during adolescence and adulthood on hippocampal neurogenesis and serotonin synthesis in rats, it was shown that treatment with fluoxetine during adolescence but not adulthood increased neurogenesis and serotonin synthesis in the vDG but not dDG (Klomp et al., 2014). Lastly, adolescent- versus adult- initiated voluntary exercise in rats had differential effects on performance on cued- and context- dependent fear conditioning with adult-initiated exercise enhancing performance on both tasks without influencing the expression of neurogenic and

plasticity markers, while adolescence-initiated exercise did not change performance on the fear conditioning tasks but enhanced expression of neurogenesis and plasticity markers (O'Leary et al., 2018). Such comparisons of treatment and ablation outcomes between adolescence and adulthood, however, need to be considered in the context of not only differences in the basal levels of neurogenesis across development, but also in terms of the hormonal and behavioural changes that occur during the adolescent period. Though limited in number, rodent studies have consistently shown that ablation of neurogenesis during adolescence results in decreased proliferation and survival of hippocampal NPCs from adolescence until late in adulthood, which also correlates with a persistent impairment in performance on memory-related tasks such as fear conditioning and the MWM test (Achanta et al., 2009; Rola et al., 2004). Similarly, human studies of children and adolescents with cancer who have had to undergo radiation therapy, have shown that such treatment is associated with lasting changes in intelligence quotient (IQ) scores and cognitive performance (Rodgers et al., 2013). Defective hippocampal neurogenesis during adolescence has been suggested as a contributing factor to the onset and development of psychiatric disorders (reviewed by (Hueston et al., 2017)), which in combination with the fact that adolescence is a period of dramatic vulnerability to the effect of extrinsic influences, means that it is imperative to expand our understanding of how positive and negative regulators of hippocampal neurogenesis such as stress and exercise influence the brain during this critical period.

1.3.3 Function of hippocampal neurogenesis during adulthood

Adult neurogenesis in the SGZ has been implicated in spatial learning and memory (Gould et al., 1999a; Kempermann, 2008; Ming and Song, 2011). Additionally, adult hippocampal neurogenesis has been implicated in the stress response as well as in the regulation of anxiety and antidepressant action (Levone et al., 2015; O'Leary and Cryan, 2014; O'Leary et al., 2012). Studying the causal link between neurogenesis and cognitive function has been enabled through the utilization of various ablation techniques such as irradiation, pharmacological interventions (with antimitotic drugs to decrease, or antidepressants to enhance neurogenesis), and transgenic mice (reviewed by (Zhao et al., 2008)).

Targeting the transiently proliferating progenitors or Type 2 cells with an antimitotic drug in adult rats resulted in impaired performance in trace conditioning task involving an eye-blink response (a hippocampal-dependent learning task; (Shors et al., 2001)), but not in other hippocampal-dependent tasks such as contextual fear conditioning and spatial learning in the MWM paradigm (Shors et al., 2002). Upon conditional deletion of TLX in the hippocampi of adult mice, which led to reduced NPC proliferation and thus reduced neurogenesis, mice exhibited impaired spatial learning and memory in the MWM task, but no change in performance on contextual fear conditioning, which is another form of hippocampal-dependent learning (Zhang et al., 2008). Interestingly, two other methods of neurogenesis ablation - irradiation and genetic ablation of NPCs - produced different results from those described above. Specifically, reducing neurogenesis with these techniques in adult mice resulted in their failure to learn the

association between a given context and receiving an electric shock (fear conditioning), while there was no deficit in their performance in the MWM task or the Y maze (Saxe et al., 2006). In addition, localized X-irradiation to the hippocampus and cortex in adult mice resulted in impaired learning in the Barnes maze task, but not the MWM task, both of which are hippocampal-dependent tasks measuring spatial memory and learning (Raber et al., 2004). What is more, adult rats that underwent irradiation also performed poorly on the fear conditioning paradigm (Winocur et al., 2006) and place learning (measured by T-maze task), but not in MWM task or NOR task (Madsen et al., 2003). Interestingly, when neurogenesis was suppressed with an antimitotic drug, adult rats showed impaired memory in the NOR test (Bruel-Jungerman et al., 2005). No effect of irradiation was observed on MWM task performance or in the novelty suppressed feeding test in adult mice (Meshi et al., 2006). On the contrary, after receiving a low dose of irradiation, adult rats displayed impaired long-term spatial memory as measured by the MWM (Snyder et al., 2005). It is interesting to note that some ablation techniques led to impaired learning of certain types of tasks, but not others. These results point to the complexity of the function of adult born neurons and also to the fact that the specific stage of neurogenesis which is targeted by the intervention can influence the specific cognitive task (Zhao et al., 2008). Furthermore, differences in the specific tests used as well as the species and strains may additionally account for the discrepancies in results. Interestingly, the treatment of brain cancer in humans often requires cranial radiation therapy which has been associated with progressive cognitive decline such as impairments in memory, attention and executive function (Sarkissian, 2005). These side effects have been attributed in part to the decrease in hippocampal neurogenesis that the treatment may

cause (Greene-Schloesser et al., 2013). Lastly, behavioural studies in rodents where neurogenesis is enhanced through enrichment have shown positive correlation between the level of adult hippocampal neurogenesis and performance on hippocampal-dependent tasks such as MWM and pattern separation (Kohman and Rhodes, 2013). Pattern separation refers to the ability to form distinct representations of similar inputs or to discriminate between similar memories and despite some discrepancies in the literature, it has largely been recognized as a hippocampal neurogenesis-dependent process (Aimone et al., 2014; Clelland et al., 2009; Deng et al., 2010; Snyder et al., 2005). Pattern separation processing can be tested in the context of fear conditioning paradigms, novel object location recognition paradigm, radial arm maze paradigm, or touchscreen operant conditioning paradigms (van Hagen et al., 2015; Yassa and Stark, 2011). Voluntary exercise, for instance, was shown to increase hippocampal neurogenesis which was coupled with enhanced performance on the spatial pattern separation paradigm novel object location and in NOR task in mice (Bolz L, 2015; Creer et al., 2010). A study where hippocampal neurogenesis was decreased using a lentiviral approach, demonstrated direct involvement of immature neurons and BDNF action in the consolidation of similar memories (Bekinschtein et al., 2014) and a meta-analysis of behavioural studies testing the same hypothesis supported the results of Bekinschtein and colleagues, highlighting only 2 studies that obtained results contradictory to the ones described above (Franca et al., 2017). To sum up, notwithstanding some discrepancies in the research thus far, a potential role of hippocampal adult-born granule cells in learning and memory appears to be important for at least some types of memory processing.

As well as being involved in learning and memory, adult neurogenesis has been shown to play a role in mood regulation and disorders related to it, such as depression (reviewed by (Balu and Lucki, 2009)). More specifically, adult hippocampal neurogenesis is involved in both the stress-response (Mirescu and Gould, 2006) and the anxiolytic response, both of which can contribute to mood dysregulation and depression (Petrik et al., 2012). However, evidence to position neurogenesis as an etiological factor in the development of mood disorders is lacking due to the fact that ablation of neurogenesis does not induce depressive-like or anxiety-like behaviours in rodents in the absence of another negative stimulus such as a stressor (Petrik et al., 2012; Zhao et al., 2008). Nevertheless, patients suffering from major depressive disorder have presented with reductions in hippocampal volume, which may be reflective of reductions in neurogenesis (Malykhin et al., 2010), and interestingly, the effectiveness of antidepressant treatment is consistent with the time that is needed for newborn cells to be integrated into the hippocampal circuitry (Malberg, 2004; Taupin, 2006). Electroconvulsive therapy, lithium and the antidepressant fluoxetine have been shown to increase the number of newborn granule cells in rodent models of depression (reviewed by (Duman et al., 2001)). For instance, using genetic and radiological methods for selectively ablating hippocampal neurogenesis, Santarelli and colleagues demonstrated that the antidepressant effect induced by fluoxetine and imipramine on the novelty suppressed feeding test (a behavioural paradigm shown to be dependent on hippocampal neurogenesis) was prevented in adult mice, as was the anti-anxiolytic effect of fluoxetine in a chronic unpredictable stress paradigm (Santarelli et al., 2003). In contrast, using a different strain of mice and X-ray irradiation for the ablation of neurogenesis, the same group of researchers showed that the effects of chronic

fluoxetine administration on measures of depressive-like behaviour such as the forced swim test (FST) and novelty-induced hypophagia were independent of hippocampal neurogenesis (Holick et al., 2008). In another strain of mice, albeit transgenic, it was found that without the presence of other external stimuli such as antidepressants or stress, the mice that were depleted of adult-born neurons presented with a significant increase in anxiety-like behaviours such as the time spent in the open arms of an elevated plus maze (EPM; (Revest et al., 2009)). Findings from rat studies appear to be more consistent in their demonstration of the necessity of newly generated granule cells for antidepressant effectiveness. This is apparent in the FST and novelty suppressed feeding test in response to X-ray irradiation-based ablation of neurogenesis (Jiang et al., 2005) and the chronic stress-induced decrease in neurogenesis-paradigm (Airan et al., 2007). Nonetheless, the precise mechanism and function of hippocampal neurogenesis in antidepressant-mediated mood regulation remains to be elucidated with many controversies arising from different species and methods used by different labs (for reviews see (Petrik et al., 2012; Zhao et al., 2008).

With respect to the potential functions of adult hippocampal neurogenesis in the human brain, the most we have learned to date comes from postmortem examination of patients who suffered from CNS disorders. Nevertheless, magnetic resonance spectroscopy has provided a useful tool for imaging NPCs in the live human brain under physiological and pathological conditions (Manganas et al., 2007). With this we have learned that neurogenesis is altered in cases of epilepsy such that seizures cause a dramatic increase in the proliferation of hippocampal NPCs and Type 3 neuroblasts

which is associated with cognitive deficits (reviewed by (Jessberger and Parent, 2015)). Furthermore, enhanced neurogenesis has been shown to follow ischemic stroke in rodents, and newborn neurons are suspected to repopulate infarct areas in human patients as well (Lindvall and Kokaia, 2015; Zhao et al., 2008). Lower levels of proliferation in the hippocampus were demonstrated in adult patients suffering from schizophrenia. The impaired cognitive function in schizophrenic patients resembles the impairments observed when hippocampal neurogenesis is inhibited (Reif et al., 2006). What is more, genetic studies suggest that the decrease in newborn cells might be important contributing factor to the development of the disease. Additionally, a recent meta-analysis suggested that the connection between neuropsychiatric disorders and dysregulated hippocampal neurogenesis is beyond epiphenomenon, and that adult-generated granule cells may hold the key to therapeutic and/or preventative strategies for alleviating suffering from these disorders (Yun et al., 2016). The processes contributing to deregulation of hippocampal neurogenesis under pathological conditions are not fully understood, but it is proposed that extracellular signals provided by the hippocampal microenvironment may interact with cell-intrinsic factors, which could present potential drug targets for the conditions listed above (Ryan and Nolan, 2016).

1.3.4 Function of hippocampal neurogenesis during ageing

Adult hippocampal neurogenesis has been proposed to be a key element in ensuring and maintaining functional hippocampal integrity in old age (Kempermann, 2015; Kempermann et al., 2002). Neurodegenerative diseases due to the age-dependent rapid and continuous loss of neurons (such as Parkinson's disease (PD) or Huntington's

disease), have been suggested to reflect the contraposition of the neurogenic process such that under homeostatic conditions a fine balance between neurodegeneration and neuroregeneration exists, and under pathological conditions the balance is disturbed and a disease manifests (Kempermann, 2015). Even though little evidence has accumulated in support of the abovementioned theory, if deemed true, it in combination with findings regarding the high potential of stem-cell-based strategies for the treatment of age-related neurodegeneration and pathologies, make the hypothesis that adult neurogenesis holds key to novel therapeutic approaches in the treatment of age-related neurodegenerative disorders rather attractive (Lindvall and Kokaia, 2015; Lindvall et al., 2004). In addition, decreased hippocampal neurogenesis is proposed as an important mechanism underlying age-related cognitive decline as well as neurodegenerative disorders such as Alzheimer's disease and various types of dementia (Kuzumaki et al., 2010). Nonetheless, the mechanisms of how hippocampal neurogenesis can function as therapeutic target for neurodegenerative conditions, remain to be further elucidated.

Similar to studies on adult hippocampal neurogenesis, the function of hippocampal neurogenesis in rodents during ageing has been studied using neurogenesis enhancing and ablating techniques in conjunction with neurogenesis-associated cognitive tasks in aged animals under normal physiological conditions. Given the positive correlation between physical activity and the reduced risk of dementia and cognitive decline in an elderly cohort (Laurin et al., 2001), the Gage lab investigated whether hippocampal neuroplasticity may account for the cellular mechanism underpinning these observed

benefits in the human study. To test their hypothesis, the authors exposed middle aged mice (10 months old) to an enriched environment, consisting of a rearrangeable set of plastic tubes, a running wheel, and nesting materials and toys for the duration of the 10 month study, a period in mice considered to reflect senescence in humans (Kempermann et al., 2002). Interestingly, the mice exposed to the enriched environment displayed a fivefold increase in the number of newborn neurons compared to controls, which was coupled with significant enhancements of their learning and memory performance on the MWM task, as well as exploratory behaviour in an open field (OF) task and locomotor activity on the rotarod. This suggests that living in a stimulating environment during ageing can induce an increase in hippocampal neuroplasticity and cognitive performance (Kempermann et al., 2002). In a study employing neurogenesis-associated behavioural tests, which probed spatial memory and pattern separation in aged rats, the authors found a positive correlation between structural alterations and neurogenesis in the hippocampus, and performance on the behavioural tests (Driscoll et al., 2006). Specifically, with the advancement of age, rats displayed decreased hippocampal volume and hippocampal neurogenesis, which was paralleled by impairments in cognitive performance on the MWM task and a pattern separation paradigm (Driscoll et al., 2006). More recently, a study in mice examined the effects of senescence on the different stages of hippocampal neurogenesis on both learning and spatial memory performance on the MWM task. The results illustrated that the decline in neurogenesis over time could best be modelled by an exponential inverted U-shape curve, such that the most rapid decline occurred between 3 and 6 months of age, after which neurogenic levels slowly but steadily decreased. Interestingly, the decrease could be accounted for in all stages of

the neurogenic process, namely proliferation, differentiation and survival. What is more, the authors found that performance in the MWM task was progressively worse with age not due to impairments in learning, but due to mice adopting more spatially imprecise strategies over time (Gil-Mohapel et al., 2013). As well as decreased neurogenesis, neurodegenerative diseases are also characterized by neuronal loss primarily due to apoptosis (Lunn et al., 2011). Upon examination of the effect of genetically induced hippocampal neuronal loss in the ageing mouse brain, it was found that apoptosis had an enhancing effect on hippocampal neurogenesis. However, despite proliferation and neuronal differentiation and survival being significantly increased, these transgenic animals performed significantly poorer on spatial memory in the Barnes maze. The results illustrated that the increase in the number of granule cells did not mitigate the cognitive deficit observed with ageing (Yeung et al., 2014). Thus, neurogenesis alone cannot account for the age-related cognitive decline observed in rodents and humans, and more mechanisms need to be taken into account for successful development of preventative and therapeutic strategies to ameliorate the deterioration of cognitive function during senescence (Kempermann, 2015).

1.4 Hippocampal neurogenesis across the septo-temporal axis

An anatomical gradient divides the hippocampus into dorsal (posterior in humans) and ventral (anterior in humans) regions, each of which is functionally distinct from each other (Tanti and Belzung, 2013). Given that hippocampal neurogenesis has been functionally implicated in rodents' performance on behavioural tasks preferentially regulated by either the dorsal hippocampus (spatial learning and memory) or the

ventral hippocampus (stress response, anxiety and depressive-like behaviour), it is reasonable to hypothesize that adult hippocampal neurogenesis may also be segregated across the septo-temporal anatomical gradient (O'Leary and Cryan, 2014).

It has been established that adult-born granule cells exhibit different features depending on their location across the dorso-ventral axis (reviewed by (O'Leary and Cryan, 2014)). For instance, the density of BLBP+ radial glia (Type 1 cells) and DCX+ immature neurons (Type 3 cells) are significantly higher in the dDG compared to the vDG of the mouse, while no difference in the numbers of transiently amplifying progenitors was observed (Jinno, 2011b). Similarly, in the adult rat dDG, neurogenesis and granule cell activation of the DG population as a whole, as measured by c-fos IHC, appeared to occur at higher rate than in the vDG (Snyder et al., 2009). Interestingly, upon examination of the activation of newborn granule cells by colocalization of c-fos and PSA-NCAM, the authors found that more adult born neurons in the vDG were activated compared to adult born neurons in the dDG (Snyder et al., 2009). Furthermore, employing retroviral labelling and IHC marker expression for the different stages of neuronal maturation in combination with patch-clamp recordings and morphological analysis, it was shown that adult born neurons in the dDG of mice matured faster than those born in the vDG (Piatti et al., 2011). This finding was replicated in adult rats, where it was shown that maturation (NeuN and immediate-early gene Arc (activity-regulated cytoskeleton-associated protein) colocalization) occurred 1-2 weeks earlier in the dDG compared to the vDG, suggesting that dorsal adult born granule cells may contribute to function sooner (Snyder et al., 2012).

Additionally, using IHC methods Jinno and colleagues showed that the decline of neurogenesis that occurs with ageing was at a significantly higher rate in the vDG than the dDG of adult mice, suggesting a possible involvement of hippocampal neurogenesis in the cognitive and affective impairments observed in the elderly (Jinno, 2011a). Of note, the embryonic origin of Type 1 NSCs from the SGZ of the DG arise from the ventral hippocampus (Li et al., 2013) which implies that NSCs of the dDG and vDG share the same intrinsic properties but are regulated by the inputs of distinct brain areas (as the connectivity of dDG and vDG is different) and respond to different extrinsic stimuli. All in all, this suggests that NSCs from the dDG and vDG are involved in separate functions (O'Leary and Cryan, 2014).

In this regard, it would be expected that behavioural tasks that recruit dorsal pathways would influence neurogenesis in dDG, and similarly, factors recruiting ventral pathways would modulate vDG neurogenesis. Training adult male rats on the MWM task (i.e. engaging animals in spatial learning) produced an increase in the number of surviving adult born neurons in the dDG and not the vDG (Ambrogini et al., 2000). Remarkably, employing another learning paradigm, trace eyeblink conditioning, showed that learning resulted in a dDG-specific increase in the number of surviving neurons of the male rats only, with female rats exhibiting increased neuronal survival in dDG but also in the vDG and at a higher magnitude when compared to sex-matched controls that did not undergo training (Dalla et al., 2009). Whether this finding reflects sex differences in the response to the task itself or whether the regulation of neurogenesis across the septo-temporal axis is distinct across males and females

remains to be determined. In addition, using another strain of male rats, researchers found an upregulation of levels of the pro-neurogenic factor BDNF in the dDG and a downregulation in the vDG, coupled with an increase in levels of postsynaptic PSD-95 in the dDG alone, after animals had undergone training at the spatial task radial arm water maze (Hawley et al., 2012). While voluntary running resulted in a uniform increase in cell proliferation, neurogenesis and maturation of newborn neurons in both the dDG and vDG of adult CD1 mice (Bednarczyk et al., 2009), it produced increased neuronal maturation in the vDG alone in adult Bl6/C57 mice (Piatti et al., 2011). Environmental enrichment has also been established as a positive regulator of hippocampal neurogenesis across the dorso-ventral axis of the mouse hippocampus (Lehmann et al., 2013; Tashiro et al., 2007). However, upon detailed examination of the different stages of neurogenesis, it transpired that while cell proliferation of transiently amplifying progenitors was increased across both dorsal and ventral SGZ, a significant increase in the surviving new neurons was only present in the dDG (Tanti et al., 2012). A recent study showed that environmental enrichment was associated with increased total hippocampal volume in mice, and distinct gene transcription in the dDG but not vDG. Differences in DNA methylation were also observed, including at the binding sites of the proneuronal transcription factor NeuroD1 in dDG but not vDG (Zhang et al., 2018). Given that exercise and enrichment have been shown to improve both cognitive performance and mood (Tanti and Belzung, 2013), and that the underlying mechanism is dependent on hippocampal neurogenesis (Schloesser et al., 2010), it is possible that the effect of exercise and enrichment is anti-anxiolytic due to increased neurogenesis in the vDG. However, because these pro-neurogenic interventions contributed to enhanced cognitive function and increased neurogenesis

in the dDG also, the mechanisms through which adult born neurons contribute to the functions of the septo-temporal axis remain to be further investigated.

Stress has been established as a prominent negative regulator of adult hippocampal neurogenesis (Schoenfeld and Gould, 2012) and has recently gained attention due to the question of the reciprocal relationship between stress and neurogenesis being restricted to the vDG (reviewed by (O'Leary and Cryan, 2014)). There was a significant reduction in the proliferation of Type 2 cells in the vDG specifically in rats that underwent chronic mild stress (Jayatissa et al., 2006) and chronic unpredictable stress (Hawley et al., 2012). Mice undergoing the unpredictable chronic stress paradigm also showed reduced proliferation in the vDG, but not the dDG. Interestingly, the mice in this study also exhibited reduced neurogenesis in the vDG in response to the stress, while another cohort exposed to environmental enrichment instead of stress, displayed increased proliferation in both dDG and vDG but only increased neurogenesis in the dDG and not the vDG (Tanti et al., 2012). When employing the same stress paradigm with the same strain of mice (BALB/c), however, another group of researchers observed reductions in cell proliferation, neurogenesis and cell survival in both dDG and vDG (Nollet et al., 2012). This finding was corroborated using maternal deprivation as a stressor in rats, namely cell proliferation, neurogenesis and neuronal survival were decreased in response to stress in both dDG and vDG (Oomen et al., 2010). While treatment with the stress hormone corticosterone resulted in overall decrease in number of proliferating cells across both the dDG and vDG of male C57BL6/J mice (Rainer et al., 2012), it preferentially downregulated

neurogenesis in the vDG of female rats. Nonetheless, male rats presented with overall decrease in number of DCX+ immature neurons across the septo-temporal DG (Brummelte and Galea, 2010).

It is well known that mice of different strains vary in their basal levels of neurogenesis, memory performance and motor coordination. Despite these genetic differences, however, neurogenic levels of mice of all strains have been shown to be positively regulated by environmental enrichment (Merritt and Rhodes, 2015). Additionally, strain differences in the responsivity to stress of male mice have been well defined, albeit the mechanisms and reasons underlying these differences have not been fully understood yet (Anisman et al., 2001; Mozhui et al., 2010; van Bogaert et al., 2006). What is more, sex differences within mouse strains (also observed in humans, reviewed by (Donner and Lowry, 2013)) and differences between females of various mouse strains in stress-related and anxiety-related behaviours have also been shown (Donner and Lowry, 2013; Marchette et al., 2018). Alongside these findings, are also data demonstrating profound differences in basal levels of hippocampal neurogenesis across the septo-temporal axis across species and strains and especially between laboratory versus wild-living rodents (Wiget et al., 2017). Thus, hippocampal neurogenesis changes in rate, function and regulation across the septo-temporal axis. However, in order to understand the distinct characteristics of this process across the dorso-ventral anatomical gradient, more studies focusing on specific sex, strain and species using well-defined neurogenesis- enhancing or inhibiting paradigms are needed.

1.5 Intrinsic pathways regulating adult hippocampal neurogenesis

1.5.1 Growth factors

Growth factors are a large family of proteins that are present in the extracellular environment and serve as signalling molecules facilitating cell development and function through binding specific transmembrane receptors on target cells (Akter, 2016). Within the nervous system, growth factors are commonly referred to as neurotrophins and are critical for progenitor proliferation, differentiation, fate determination and neuronal survival. Growth factors participate in the regulation of these processes within the neurogenic niches of the brain from embryonic development through adulthood and ageing (Friedman, 2012). A number of growth factors have been shown to participate in the maintenance of adult hippocampal neurogenesis, namely: IGF-1 and insulin-like growth factor 2 (IGF-2); FGF2; VEGF; BDNF; neurotrophin 3 (NT-3; (Faigle and Song, 2013)).

1.5.1.1 IGFs

Peripheral administration of IGF-1 to the adult rat increased NPC proliferation in the hippocampus after 6 days, which resulted in increased number of newborn neurons but not astrocytes 14 days later (Aberg et al., 2000). Interestingly, treating primary cultures of adult rat hippocampal NPCs with IGF-1 at high and low doses induced increased cell proliferation and neuronal differentiation, respectively (Aberg et al., 2003). Furthermore, levels of endogenous IGF-1 decreased with age in rats, which was

coupled with a decrease in hippocampal neurogenesis. Administration of IGF-1 through i.c.v. infusion restored NPC proliferation and neurogenesis in the hippocampi of two-year-old rats (Lichtenwalner et al., 2001). Lastly, more recent evidence from transgenic mice specifically overexpressing IGF-1 in NPCs, illustrated that IGF-1 positively regulates NPC proliferation and differentiation through two autonomous downstream pathways, thereby confirming *in vitro* data that IGF-1 can exert its effects on the distinct phases of the neurogenic process in a dose- and pathway- dependent manner (Aberg et al., 2003; Yuan et al., 2015). IGF-2 on the other hand has been shown to regulate NPC proliferation but not differentiation in the hippocampus of adult mice. Interestingly, knockdown of IGF-2 selectively reduced the number of proliferating cells in the SGZ, while proliferation rate in the SVZ remained the same (Bracko et al., 2012).

1.5.1.2 FGF

An elegant study employing a complex design of independently blocking or enhancing FGF signalling in the hippocampus of adult mice, illustrated that interaction between the growth factor and its receptors is necessary for the maintenance of the NSC pool within the adult DG (Kang and Hebert, 2015). Interestingly, impaired FGF signalling did not influence NPC proliferation and differentiation, however, enhancing FGF signalling led to increased NPC proliferation and neurogenesis in the hippocampi of 3- to 4-month-old mice (Kang and Hebert, 2015). What is more, enhanced FGF signalling reversed the decline in neurogenesis observed in ageing mice (13- to 15-month-old) (Kang and Hebert, 2015). These data corroborated findings from ageing rats, which showed that i.c.v. infusions of FGF-2 not only restored the age-related

decrease in neurogenesis, but also enhanced the dendritic growth and complexity of hippocampal newborn neurons (Rai et al., 2007).

1.5.1.3 VEGF

Next to being an angiogenic protein (Ogunshola et al., 2002), VEGF also exhibits neurotrophic and neuroprotective properties by stimulating proliferation of vascular endothelial cells (Sondell et al., 1999; Sondell et al., 2000). Infusion with VEGF to the SGZ of the adult rat resulted in increased number of proliferating progenitors, newborn neurons and astrocytes, but no change in number of apoptotic cells (Jin et al., 2002). Remarkably, administration of antidepressants in adult rats induced VEGF expression in a time-dependent manner, which was coupled with the stimulation of hippocampal NPC proliferation and therapeutic action of antidepressants as measured by behavioural testing (Warner-Schmidt and Duman, 2007). These growth factors (IGFs, FGF and VEGF) have been shown to exert their effects on hippocampal neurogenesis through common signalling mechanisms, namely they bind to a ligand-specific receptor from the tyrosine kinase family, which results in a cascade of events that activate the downstream signalling pathways PI-3 kinase/Akt and the Ras/Raf/Mek/Erk pathway (Faigle and Song, 2013). For example, IGF-1 exerts its differential effect through activation of the PI-3 kinase/Akt for inducing NPC proliferation and activation of the Ras/Raf/Mek/Erk pathway for inducing differentiation (Aberg et al., 2003).

1.5.1.4 BDNF

The neurotrophins BDNF and NT-3 also bind to receptors from the tyrosine kinase family, namely BDNF binds to Tropomyosin receptor kinase B (TrkB) and NT-3 binds to Tropomyosin receptor kinase C (TrkC), while both also bind to p75 neurotrophin receptor (p75NTR) (Faigle and Song, 2013). Direct infusion of BDNF into the hippocampi of adult rats led to a marked increase in the number of BrdU+/NeuN+ cells. However, worthy of note is that neurogenesis had not only increased in the neurogenic niche of the DG, the GCL, but also in the hilus, where ectopic newborn granule neurons were identified (Scharfman et al., 2005). Additionally, selective silencing of the BDNF receptor TrkB within mouse hippocampal NPCs alone, resulted in decreased cell proliferation, premature exit of cell cycle and thereby a decrease in adult hippocampal neurogenesis. Moreover, silencing of the receptor blocked the increase in cell proliferation in the DG following environmental enrichment or antidepressant treatment, rendering these mice behaviourally insensitive to the beneficial effects of either of those interventions (Li et al., 2008d). Using a similar approach, another group illustrated that 4 weeks after silencing the TrkB receptor in NPCs of the DG of adult mice, newborn neurons exhibited a reduction in dendritic length, arborisation and spine density. Furthermore, the researchers showed that intact BDNF-TrkB signalling was necessary for the recruitment of adult-born neurons to functional connections, as well as the neurogenesis-induced LTP and survival of these cells (Bergami et al., 2008). Lastly, two transgenic mouse models were used to illustrate that the enriched-environment-induced increase in cell proliferation and neurogenesis in the adult hippocampus is mediated through BDNF specifically, as opposed to another neurotrophin-4 (NT-4) that binds to the same TrkB receptor (Rossi

et al., 2006). BDNF is the most extensively studied neurotrophin in the context of adult hippocampal neurogenesis.

1.5.1.5 NT-3

NT-3 has been suggested as another neurotrophic diffusible factor and excellent candidate for the regulation of hippocampal neurogenesis (Faigle and Song, 2013). The expression of NT-3 in the adult rat brain has been shown to be restricted primarily to the DG (Friedman et al., 1991; Lauterborn et al., 1994). Furthermore, it has been shown that administration of NT-3 to primary cultures of hippocampal NPCs reduced their proliferation and stimulated their differentiation towards neurons (Vicario-Abejon et al., 1995). What is more, in primary cortical cultures from rats, NT-3 administration antagonized the proliferative effects of FGF (Ghosh and Greenberg, 1995). *In vivo,* NT-3 knockout mice exhibited a reduced number of proliferating cells as well as a reduced number of surviving adult born neurons in the DG, while no change in the number of newly born surviving astrocytes was observed as measured by BrdU/GFAP immunolabeling (Shimazu et al., 2006).

To sum up, growth factors and neurotrophins exert their effect on adult hippocampal neurogenesis by stimulating NPC proliferation or inducing neuronal differentiation.

1.5.2 Neurotransmitters

Neurotransmitters are conventionally defined as endogenous small diffusible chemicals that facilitate communication between neurons by being released from

synaptic vesicles into the synaptic cleft and binding to their respective receptors on the target cell (González-Espinosa and Guzmán-Mejía, 2014). Depending on the receptors present on the target cells, neurotransmitters can cause inhibition and/or excitation (Deutch, 2013).

1.5.2.1 Glutamate

Glutamate, an excitatory chemical messenger, is the most abundant neurotransmitter in the vertebrate nervous system and is present in more than 90% of the synaptic connections in the human brain (Meldrum, 2000). Glutamatergic signalling has been suggested to play an important role in processes such as learning and memory (McEntee and Crook, 1993) especially due to its involvement in synaptic plasticity and LTP (Kullmann and Lamsa, 2008; Malenka and Bear, 2004). Glutamate exerts its functions through binding to several different classes of receptors – the ionotropic N-methyl-D-aspartate receptor (NMDAR), the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), kainic acid receptor, and the metabotropic glutamate receptors (Faigle and Song, 2013). In the past it was shown that pharmacological blockade of the NMDA receptors in the developing rat DG resulted in increased cell proliferation and apoptosis coupled with a decreased number of functionally integrated neurons (Gould et al., 1994). Furthermore, neurogenesis in the adult rat DG was shown to be regulated by excitatory input and NMDA receptor activation (Cameron et al., 1995). More recent studies have explored the precise mechanisms and effects that glutamatergic signalling exerts on adult hippocampal neurogenesis. A thorough analysis of NMDAR subunit expression in different cell types of the rat DG revealed the presence of the subunits NR1 and NR2B on GFAP+

radial-glia-like Type 1 cells (NSCs) but not on NPCs of Type 2a and 2b or Type 3 (Nacher et al., 2007). Interestingly the expression of these subunits re-emerged on newly born neurons that were 14 days old suggesting involvement of an excitatory pathway where NR1 and NR2B activation may play a role in NSC proliferation and adult born neuronal survival (Nacher et al., 2007). An *in vitro* study employing a wide array of approaches to address mechanisms underlying the relationship between NMDAR activation and neurogenesis illustrated that an intrinsic property of proliferating adult hippocampal NPCs was a coupling between excitation and neurogenesis, such that NMDAR excitation was associated with a decrease in expression of the glial fate genes *Hes1* (hairy and enhancer of split-1) and *Id2* (DNA-binding protein inhibitor 2), and an induction of expression of the proneuronal transcription factor NeuroD (Deisseroth et al., 2004). Furthermore, an elegant *in vivo* study using a retrovirus-mediated single-cell gene knockout technique showed that the survival of new neurons and the consequent formation of functional circuits within the hippocampus are depend on the input-specific and cell-specific activity of NMDARs within the critical period of them being expressed on newly born neurons (Tashiro et al., 2006). Investigation into the regulation of adult hippocampal neurogenesis through other receptors such as the AMPARs and kainic acid receptors, converge on the data obtained from NMDARs, positioning glutamate as an important regulator of adult born neuronal survival (Bai et al., 2003; Jessberger et al., 2007).

1.5.2.2 GABA

GABA is the principal inhibitory neurotransmitter in the adult mammalian nervous system (Watanabe et al., 2002) and the primary excitatory neurotransmitter in the

developing brain (Ben-Ari et al., 2007). GABA binds to two general classes of receptors: the ionotropic $GABA_A$ receptor, binding to which forms a ligand-gated ion channel complex permeable to chloride (Cl^-); and the metabotropic $GABA_B$ receptors, binding to which results in coupling to G proteins (Hamill et al., 1983). Within the adult nervous system, activation of $GABA_A$ receptors allows for Cl^- to flow into the cell, which hyperpolarizes the membrane and therefore inhibits action potential (Li and Xu, 2008). The intracellular concentration of chloride ($[Cl^-]_i$) is different across the neurons of the developing and adult nervous system, with high $[Cl^-]_i$ in immature and low $[Cl^-]_i$ in mature neurons, respectively (Succol et al., 2012). Thus, activation of $GABA_A$ receptors in the developing nervous system leads to efflux of Cl^- ions from the cell, which depolarizes the membrane and triggers an action potential (Ben-Ari et al., 2007; Li and Xu, 2008). During development, a switch occurs which causes the change of intracellular $[Cl^-]_i$ in neurons rendering GABA an inhibitory neurotransmitter (Faigle and Song, 2013). With respect to adult hippocampal neurogenesis, retroviral labelling of mouse newborn granule neurons combined with electrophysiological recordings of the cells showed that ambient GABA tonically activated these cells before they became sequentially innervated by GABA- and glutamate-mediated synaptic inputs (Ge et al., 2005). Furthermore, the same group of researchers showed that converting GABA from an excitatory to an inhibitory neurotransmitter *in vivo* by changing the intracellular $[Cl^-]_i$ of immature hippocampal neurons, caused these cells to fail to form functional synapses (Ge et al., 2005). Next to promoting the integration of adult-born hippocampal neurons, GABA has been shown to maintain hippocampal NSCs in their quiescent state (Song et al., 2012). Specifically, the tonic input of parvalbumin-expressing local neurons to radial glia-

like NSCs within the mouse hippocampus was shown to inhibit their activation in a GABA-mediated manner. *In vivo* impairment of GABA signalling between the interneurons and hippocampal NSCs through viral knockdown of a specific subunit of the $GABA_A$ receptor led to rapid exit of quiescence and enhanced symmetric self-renewal of the NSCs (Song et al., 2012). To sum up, GABA affects the stages of neurogenesis in a two-fold manner: while it maintains NSCs in their quiescent state, it promotes the differentiation and survival of newborn neurons.

1.5.2.3 Serotonin

Serotonin or 5-hydroxytryptamine (5-HT) is a monoamine neurotransmitter involved in the regulation of appetite (Feijó et al., 2011), mood (Wallin and Rissanen, 1994) and sleep (Jouvet, 1999). Attention to the serotonergic effects on hippocampal neurogenesis was drawn after establishing its relationship with stress and depression (Gould, 1999), which coincided with the discoveries that the DG is enriched with 5-HT_{1A} type of receptors (Azmitia et al., 1996) and that the DG receives serotonergic-mediated input from the median raphe nucleus of the brainstem (Patel et al., 1996). When examining the direct effects of serotonin on adult hippocampal neurogenesis through blockade or activation of various types of 5-HT receptors, it was shown that serotonin transmission increases the proliferation of NPCs and can increase neurogenesis in the SVZ and the olfactory bulb (Banasr et al., 2004; Radley and Jacobs, 2002).

1.5.2.4 Acetylcholine

Acetylcholine (Ach) is a neurotransmitter present in areas of the brain involved in arousal (Van Dort et al., 2009), attention (Himmelheber et al., 2000), memory (Hasselmo and McGaughy, 2004) and motivation (Brunzell and McIntosh, 2011; Pratt and Kelley, 2004). The DG receives cholinergic innervations from areas of the forebrain (Aimone et al., 2014) which made the neurotransmitter an interesting potential regulator of hippocampal neurogenesis. Indeed, depleting Ach by lesioning the basal forebrain cholinergic regions in the adult rat, led to a decrease in neuronal survival and an increase in apoptosis in the SGZ and olfactory bulb (Cooper-Kuhn et al., 2004). What is more, pharmacological increase of the levels of Ach in the adult mouse resulted in an increase in neuronal survival 5 weeks post intervention and no change in the number of proliferating NPCs (Kaneko et al., 2006).

1.5.2.5 Dopamine

Dopamine is a neurotransmitter from the catecholamine and phenethylamine families, which is synthesized in dopaminergic neurons in the brain and in cells in the medulla of the adrenal glands (Kim et al., 2016a). Dopaminergic signalling in the brain has been shown to play key roles in motor control (Joshua et al., 2009), reward-motivated behaviour (Berridge and Robinson, 1998; Berridge et al., 2009) and executive function and cognitive control (Saddoris et al., 2015). Impairments in dopaminergic pathways have been linked to psychiatric disorders such as schizophrenia (Dean and Hussain, 2001; Kestler et al., 2001), depression (D'Aquila et al., 2000; Meyer et al., 2001), attention deficit hyperactivity disorder (ADHD) (Volkow et al., 2009), addiction (Di Chiara, 1999) and neurodegenerative diseases such as PD (Mattammal et al., 1995),

Huntington's disease (Bernheimer et al., 1973) and dementias (Luo and Roth, 2000). In rodent models of PD, where dopamine is depleted, NPC proliferation in the SGZ and SVZ was decreased, an effect rescued by subsequent stimulation of dopamine receptors (Höglinger et al., 2004). Furthermore, post-mortem immunohistochemical analysis of the brains of PD patients revealed a decrease in the number of NPCs in these neurogenic niches (Höglinger et al., 2004). These data suggest that the dopaminergic afferents originating in the ventral tegmental area and innervating the hippocampus play a role in the stimulation of NPC proliferation in the SGZ (reviewed by (Faigle and Song, 2013).

1.5.2.6 Noradrenaline

Noradrenaline is a neurotransmitter of the catecholamine family and is produced by the locus coeruleus nuclei of the pons (Harley, 2007). Noradrenaline, released at high levels during wakefulness and low levels during sleep (Linsell et al., 1985), is important for arousal and alertness (Mather et al., 2016; Oken et al., 1995), boosts vigilance and enhances focused attention (Oken et al., 2006). Noradrenaline can further promote memory formation and retrieval (Roozendaal, 2002; Roozendaal, 2003), but also induces restlessness and anxiety (Bandelow et al., 2012). Given the strong noradrenergic innervation of the adult hippocampus (Harley, 2007) a number of studies examined its role in adult neurogenesis. While selective depletion of noradrenaline decreased NPC proliferation without inducing changes in the rate of differentiation or the number of surviving granule neurons in the adult rat hippocampus (Kulkarni et al., 2002), the increase in noradrenaline release through blockade of noradrenaline-axon autoreceptor inhibition resulted in increased neuronal

survival through decreased apoptosis. In addition, no change in the proliferation of NPCs and enhanced dendritic complexity and density of newly born (PSA-NCAM+) neurons was observed (Rizk et al., 2006). Only a limited number of studies investigating the role of noradrenaline in regulation of hippocampal neurogenesis have emerged yet, thus no firm conclusions about direction or mechanism of action can be drawn. In an *in vitro* system of adult rat hippocampal Type 2a progenitors it was shown that noradrenaline administration exerted enhancing effects on the proliferation of these cells (Masuda et al., 2012), but further research is warranted to better understand the regulation of adult neurogenesis by local circuit factors such as neurotransmitters.

1.5.3 Morphogens

Morphogens are signalling molecules that originate from a specific region of the brain or another organ of the body and spread away from their source forming a concentration gradient. This concentration gradient serves as a guiding signal for various types of cells during development to ensure that they adopt a certain fate and position in the brain or body thereby prefiguring a specific pattern of development (Tabata and Takei, 2004). Morphogens involved in brain development and adult hippocampal neurogenesis are members of the Shh family, the Notch homologue family, the Wnt signalling pathway, and the bone morphogenic protein (BMP) family (Faigle and Song, 2013).

1.5.3.1 Notch signalling

The Notch signalling pathway is a highly conserved cell signalling mechanism involved in a multitude of processes within the developing nervous system, including

cell proliferation, differentiation and apoptosis (Artavanis-Tsakonas et al., 1999; Pierfelice et al., 2011; Yoon and Gaiano, 2005). The Notch signalling pathway facilitates cell-cell signalling between neighbouring cells, through ligands of the Delta and Jagged gene families that bind to the Notch receptors, of which four are known in the mammalian system (Notch1-Notch4) (Faigle and Song, 2013). Receptors and ligands alike are single-pass transmembrane proteins, which upon binding to each other cleave the transmembrane domain which results in the release of the Notch intracellular domain (NICD) from the plasma of the membrane to the cytosol (Yoon and Gaiano, 2005). NICD subsequently translocates to the nucleus where it forms an activator complex with other proteins and initiates expression of transcription factors and target genes such as the *Hes*-related protein genes. These are basic helix-loop-helix (bHLH) transcriptional regulators that inhibit expression of proneural genes and the neurogenins (Yoon and Gaiano, 2005). This positions the Notch signalling pathway as a central regulator of the inhibition of NSC differentiation and has been shown as such in the developing nervous system (Bray and Bernard, 2010). Within the murine postnatal DG where Notch and NICD are expressed from birth to adulthood, Notch signalling was shown to act as a binary switch for NSCs shifting to NPCs through symmetric division. This was illustrated through a Notch1 receptor gain- and loss-of-function experimental design, such that *in vivo* depletion of Notch1 resulted in NSCs exit of cell cycle and commitment to NPC or neuronal fate, and activation of Notch1 contributed to NSCs proliferation (Breunig et al., 2007). Additionally, Breunig and colleagues showed that Notch signalling played a role in enhancing dendritic morphology and arborization development as well as in the survival of newborn granule cells (Breunig et al., 2007). These data were supported in another study, where

the ligand for Notch1 – Jagged1 was conditionally knocked down from the mouse DG during different stages of development – embryonic, adolescent and adult (Lavado and Oliver, 2014). Jagged1 deletion during embryonic development did not lead to impairments in the formation of the DG. Mice lacking Jagged1 during adolescence, however, displayed a smaller DG and knockdown of Jagged1 during adulthood resulted in a transient increase in hippocampal neurogenesis, followed by depletion of the NSC pool (Lavado and Oliver, 2014). Recombinant binding protein suppressor of hairless (RBPJ) is one of the proteins that NICD forms an activator complex with at the cell nucleus. Conditional deletion of RBPJ in the adult mouse DG resulted in the same phenomenon as observed for Jagged1 deletion – transient increase in neurogenesis and depletion of the hippocampal NSC pool over time (Imayoshi et al., 2010). This thus confirms the importance of Notch signalling for adult hippocampal neurogenesis.

1.5.3.2 Wnt signalling

The Wnt signalling pathway is activated by Wnt ligands, which are secreted signalling molecules that bind to one or more of the seven-pass transmembrane receptors of the Frizzled (FZD) family. In mammals, the Wnt ligands comprise a family of 19 glycoproteins and each one can activate the canonical (Wnt/β-catenin) or non-canonical (Wnt/Ca^{2+} or Wnt/PCP) Wnt signalling cascades (Inestrosa and Varela-Nallar, 2015). Activation of the canonical Wnt/β-catenin pathway leads to inhibition of the activity of enzymes, such as glycogen synthase kinase 3 beta (GSK3β), which would phosphorylate β-catenin for proteasomal degradation. This results in the accumulation of β-catenin in the cytosol and its subsequent translocation to the

nucleus, where it interacts with several transcription factors and thereby regulates Wnt target genes' expression (Nusse and Varmus, 2012). Activation of the non-canonical planar cell polarity pathway (Wnt/PCP) leads to activation of the small GTPases Rho and Rac and subsequently the downstream Jun N-terminal kinase (JNK) (Gordon and Nusse, 2006). This pathway is involved in the regulation of cytoskeleton dynamics (Inestrosa and Varela-Nallar, 2015). Activation of the non-canonical Wnt/Ca^{2+} pathway is mediated through activation of G-protein coupled receptors and it regulates the Ca^{2+} concentration within the cell (Inestrosa and Varela-Nallar, 2015). The Wnt signalling pathways regulate important facets of embryonic development such as fate specification, polarity and migration (Nusse and Varmus, 2012). After confirming the expression of several receptor members of the canonical Wnt signalling pathway within the DG of adult female rats, a study further showed that inhibiting the pathway both *in vitro* (in adult hippocampal NPC cultures) and *in vivo* resulted in a marked reduction of neurogenesis. Enhancing Wnt signalling in both systems had the opposite effect in that it increased the numbers of DCX+ cells (Lie et al., 2005). While Lie et al., did not examine the specific effect of Wnt signalling on hippocampal NSC proliferation and differentiation, another group of researchers set out to investigate the mechanism of within-niche Wnt signalling using a novel *in vitro* system for both adult rat hippocampal NPCs and human NPCs. Using a series of experiments where cultures were treated with Wnt agonists or antagonists, the authors established that inhibiting endogenous canonical Wnt signalling leads to increased NPCs neuronal differentiation in both human and rat NPC cultures. This supports a model in which Wnt signalling plays a role in NS/NPCs maintenance by promoting cell-cycle re-entry and repressing differentiation (Wexler et al., 2009). Lastly, a study in adult male rats where

hippocampal neurogenesis was downregulated by inhibiting Wnt signalling within the DG, resulted in impaired performance on neurogenesis-associated tasks such as spatial memory in the MWM and recall in NOR, but not in hippocampal tasks independent of neurogenesis such as social transmission of food preference (Jessberger et al., 2009). We and others have shown that the regulation of hippocampal NSCs maintenance by canonical Wnt signalling can also be mediated through TLX-dependent Wnt activation (Green and Nolan, 2012b; Qu et al., 2009).

1.5.3.3 BMP signalling

BMPs are a group of extracellular signalling molecules with more than 20 members identified to date and belong to the transforming growth factor beta (TGF-β) superfamily of cytokines (Faigle and Song, 2013). BMP signalling is important for the regulation of both peripheral and CNS development. Within the CNS, BMP signalling plays a role in the coordination of cell fate, proliferation and differentiation during critical periods of development of the various CNS regions (Liu and Niswander, 2005). Furthermore, a role for BMP signalling in roof plate repulsion of commissural axons has also been established during embryonic development (Augsburger et al., 1999; Butler and Dodd, 2003). As with the other morphogens discussed thus far, BMP signalling is activated once a BMP ligand binds to the two transmembrane BMP receptors, namely BMPR1 and BMPR2. This in turn leads to the phosphorylation of SMAD (homologies to the Caenorhabditis elegans SMA "small" worm phenotype and Drosophila MAD "Mothers Against Decapentaplegic") proteins which form a complex and enter the nucleus. There they regulate promoter activity together with transcriptional co-repressors and co-activators, thereby exercising negative or positive

control over gene expression, respectively (Liu and Niswander, 2005). The function of BMP signalling within the adult mouse hippocampus has been studied with the aid of lentiviral vectors overexpressing BMP ligands or knocking down BMP receptors in a cell-specific manner, as well as with the use of i.c.v. infusion of the BMP antagonist Noggin (Faigle and Song, 2013). These studies have shown that BMP signalling is active in nondividing adult hippocampal NSCs and newly born neurons, such that *in vitro* treatment of NSCs with BMP enhanced the quiescence of hippocampal NSCs and *in vivo* inhibition of BMP signalling led to activation of quiescent NSCs and a transient increase in neurogenesis (Mira et al., 2010). Furthermore, it was demonstrated that BMP signalling in the SGZ regulates the pace of NPC maturation by orchestrating their quiescent/active shift during multiple stages along the lineage, thereby providing mechanism for rapid expansion of newborn neuron numbers in response to environmental stimuli such as exercise and learning (Bond et al., 2014). Additionally, BMP signalling has been shown to regulate postnatal astrogenesis by inhibiting the expression of neurogenic promoters (Qin et al., 2014). To sum up, BMP signalling in the adult hippocampal DG is important for NSC maintenance, the tempo of their maturation and proliferation, as well as maintaining the equilibrium between glial and neuronal differentiation of NPCs (Faigle and Song, 2013).

1.5.3.4 Sonic Hedgehog signalling

Shh is one of the best established examples of a morphogen and was first identified four decades ago in the fruit-fly Drosophila melanogaster (Faigle and Song, 2013; Nüsslein-Volhard and Wieschaus, 1980). The soluble signalling protein is part of the hedgehog signalling pathway, with Shh being the most intensely studied hedgehog

ligand in the vertebrate system (Placzek and Briscoe, 2018). Shh plays critical role in development guiding the patterning of different systems in the body and was first recognized as an important morphogen in the development of the zone of polarizing activity at the posterior margin of the limb bud (Riddle et al., 1993). Shh signalling has later been demonstrated to be involved in the vertebrate neural tube development (Placzek and Briscoe, 2018) and more specifically Shh was shown to induce the differentiation of ventral forebrain neurons, positioning it as the main signal for ventral patterning of the neural tube (Ericson et al., 1995). Additionally, Shh signalling plays an important role in the induction of midbrain dopaminergic neurons (Hynes et al., 1995) as well as in the maintenance of long-lasting proliferation of neuronal precursors in the cerebellum (Wechsler-Reya and Scott, 1999) and in the proliferation and survival of NPCs in the developing brain (Britto et al., 2002). In the adult rat hippocampus, the protein patched homolog 1 (PTCH1) which is Shh transmembrane receptor on the target cell, was found to be expressed by NPCs and adeno-associated viral (AAV) delivery of Shh directly to the hippocampus induced a marked increase in NPCs proliferation (Lai et al., 2003). Using an *in vivo* genetic fate-mapping approach adopting Gli1 (glioma-associated oncogene) as an output for Shh activity, Ahn and Joyner showed that the neurogenic niches in the SGZ and SVZ are established sequentially from radial glia during the late stages of embryonic development guided by Shh signalling. Furthermore, the quiescent and active NSCs as well as the NPCs in these areas continue to respond to Shh signalling later in adulthood (Ahn and Joyner, 2005). Finally, genetic ablation of protein Smoothened (SMO) which is a downstream target of PTCH1 activation, during embryonic mouse development resulted in a lack of postnatal DG neurogenesis and a severely hypotrophic hippocampus during

adulthood, illustrating another pathway through which Shh signalling contributes to the expansion and establishment of adult hippocampal neurogenesis (Han et al., 2008).

1.5.4 Epigenetic factors

Epigenetic processes such as DNA methylation, histone modification, chromatin remodelling and transcriptional feedback loops take place within the cell and bring about continual biological changes without affecting the genomic sequence (Ma et al., 2010). The different factors that take part in epigenetic mechanisms and can thereby regulate adult hippocampal neurogenesis are outlined here.

1.5.4.1 MBD1

Methyl-CpG-binding domain protein 1 (MBD1) is a protein that binds to methylated DNA sequences and thus regulates gene transcription. It has also been shown to influence repression of gene expression through chromatin remodelling (Cross et al., 1997; Hendrich et al., 1999). Mice lacking the MBD1 gene from embryo developed normally but displayed decreased hippocampal neurogenesis and impaired hippocampal function as measured by a spatial learning task during adulthood (Zhao et al., 2003). Additionally, when measuring LTP of granule cells in the DG, the researchers discovered a significant reduction in LTP response in MBD1$^{-/-}$ mice compared to their wildtype littermates (Zhao et al., 2003). Subsequently, mechanisms of action of MBD1 in the control of adult neurogenesis were revealed using two different *in vitro* systems. In NSPCs isolated from adult rat cortical tissue, it was demonstrated that MBD1 is involved in the methylation of the FGF2 promoter, thereby downregulating FGF2 expression and promoting the neuronal differentiation of the

NSPCs (Li et al., 2008b). Moreover, in NSPC cultures isolated from hippocampal tissue of either MBD1$^{-/-}$ or wildtype (WT) mice it was shown that maintaining the balance between NSPC proliferation and neuronal differentiation was dependent on MBD1 downregulation of the proliferation-promoting miR-184 (Liu et al., 2010a).

1.5.4.2 MeCP2

Methyl-CpG-binding protein 2 (MeCP2) transcriptionally represses gene expression by binding to methylated DNA sequences. It presents a similar pattern of expression as MBD1, primarily on neurons of the mammalian CNS, and is densely expressed in the adult rodent hippocampus (Kohyama et al., 2008; Shahbazian et al., 2002; Zhao et al., 2003). Interestingly, in a transgenic MeCP2 knockout (MeCP2^{KO}) mouse model, the number of adult born neurons within the DG remained the same as the number of newborn neurons observed in wildtype animals. However, the adult born hippocampal neurons of MeCP2^{KO} mice exhibited significant impairments in their maturation, namely an abnormal expression of presynaptic proteins and a diminished number of dendritic spines coupled with downregulation of large number of genes involved in synaptogenesis (Smrt et al., 2007). These data display a role for MeCP2 in maintaining an orderly maturation process for the adult born hippocampal granule cells. Nonetheless, another function of MeCP2 is to promote neuronal identity and phenotype in NPCs by initiating methylation of the GFAP promoter thereby inhibiting its expression during the period the cell is undergoing neuronal maturation (Kohyama et al., 2008). This result was supported by studies in embryonic *in vitro* and *in vivo* murine systems, showing that overexpression of MeCP2 resulted in induction of neuronal differentiation while also suppressing astroglial differentiation of NSCs

(Tsujimura et al., 2009). Additionally, a series of experiments with NSC isolates from adult MeCP2^{KO} mice illustrated that MeCP2 can inhibit NSC proliferation and promote their differentiation into neurons. This would account for the fact that no decrease in neurogenesis was found in the hippocampi of MeCP2^{KO} mice (Smrt et al., 2007; Szulwach et al., 2010). What is more, the authors elegantly demonstrated that MeCP2 together with SOX2 transcriptionally represses expression of miR-137. Thus, these studies confirm that homeostasis between NSC proliferation and differentiation is epigenetically regulated (Szulwach et al., 2010).

1.5.4.3 HDACs

Histone deacetylases (HDACs) are a class of enzymes that strip off the acetyl groups from ε-N-acetyl lysine amino acids on histones, while histone acetylases (HATs) add them. These processes affect how tightly wrapped the DNA sequence is by the surrounding histones. Expression of DNA is then controlled by acetylation, an increase in gene expression, and deacetylation, a decrease in gene expression (Yang and Seto, 2007). In the context of adult hippocampal neurogenesis, both *in vivo* and *in vitro* inhibition of HDACs with valproic acid (VPA) resulted in a decrease in NPC proliferation and an induction of neuronal differentiation even in the case of pro-astrocytic and pro-oligodendrocytic lineage-favouring factors present in the culture media (Hsieh et al., 2004). What is more, the authors showed that inhibiting HDACs resulted in an upregulation of the pro-neurogenic marker NeuroD, implicating HDAC regulation of adult hippocampal NPCs proliferation and differentiation through inhibition of NeuroD expression (Hsieh et al., 2004). In an *in vitro* murine NSC culture, it was elegantly shown that the nuclear receptor TLX recruits HDAC3 and

HDAC5 to transcriptionally repress genes promoting NSC exit of cell cycle. Thus, the authors effectively showed that HDACs play a role as TLX' transcriptional corepressors to maintain stem cells in their proliferative and non-differentiative state (Sun et al., 2007). Interestingly, another member of the HDAC family – HDAC2, was shown to regulate adult, but not embryonic, neurogenesis based on characterization of brain development of HDAC2-deficient mice. Remarkably, only the adult hippocampal neurogenic niche was affected in this knockout model, such that neuronal differentiation and survival were impaired, possibly due to disproportionate increase in NPC proliferation (Jawerka et al., 2010). To sum up, HDACs are important epigenetic regulators of the hippocampal NSC pool.

1.5.4.4 MicroRNAs

MicroRNAs (miRNAs) are a large family of small (20-22 nucleotides) non-coding RNA molecules that regulate gene expression post-transcriptionally resulting in messenger RNA (mRNA) degradation or translation inhibition (Sun and Shi, 2015). A wide range of biological processes related to cell development are regulated by miRNAs (Gao, 2010). For instance, it has been shown that in the context of neurogenesis, miRNAs regulate not only NSCs proliferation (Shi et al., 2010), but also their differentiation and fate specification (Lang and Shi, 2012; Li and Jin, 2010). miRNAs also play a role in neuronal maturation, process outgrowth and the morphological arborization as well as apoptosis of newly born cells (Gao, 2010; Sun and Shi, 2015).

While some miRNAs are found everywhere in the body, others present a developmental stage or tissue-specific expression (Pasquinelli et al., 2005). Among these are miR-137 and miR-184, which have been shown to be particularly enriched in the brain (Nomura et al., 2008; Sun et al., 2011a). miR-184 forms a regulatory network with Numbl (NUMB like; endocytic adaptor protein) to control the balance between symmetric and asymmetric division of hippocampal NSCs under the epigenetic regulation of MBD1 (Liu et al., 2010a). miR-137 was shown to be co-repressed by MeCP and SOX2 *in vitro* to induce neuronal differentiation (Szulwach et al., 2010). In the hippocampi of wildtype mice as well as *in vitro* in cultures of isolated hippocampal NSCs, it was shown that overexpression of miR-137 resulted in impaired neuronal maturation, while silencing the expression of miR-137 had the opposite effect (Smrt et al., 2010). More recently, it was shown that miR-137 regulates the fate specification of embryonic NSCs, inhibits their proliferation and promotes their differentiation into neurons (Sun et al., 2011a). What is more, the authors demonstrated that TLX represses miR-137, while miR-137 in its own right represses the expression of the histone lysine-specific demethylase 1 (LSD1) which in turn represses TLX expression. This regulatory loop is responsible for maintaining balance between embryonic NSCs proliferation, differentiation and fate determination (Sun et al., 2011a). Similarly, another brain-specific microRNA, miR-219, was shown to form a regulatory cascade with TLX, whereby an increase in miR-219 expression led to a decrease in NSC proliferation and is also associated with a decrease in TLX expression (Murai et al., 2016). While miR-219 performs these actions downstream of TLX in homeostatic conditions, TLX downregulates miR-219 to maintain a balanced proliferation/differentiation outcome for NSCs (Murai et al., 2016).

The *lethal-7* (*let-7*) gene, identified from the study of developmental timing in *Caenorhabditis elegans (C. elegans)*, is one of the first non-coding RNA molecules to be discovered (Reinhart et al., 2000; Rougvie, 2001). The *let-7* family is highly conserved across species both structurally and functionally and was the first known human miRNA (Pasquinelli et al., 2000). Three members of the *let-7* family have been implicated in the control of NSC proliferation through TLX suppression, namely miRNA-let7a (Song et al., 2015; Song et al., 2016) and miR-let7d (Zhao et al., 2013a) in embryonic cultures and miR-let7b in adult NSC cultures (Zhao et al., 2010). Another interesting miRNA candidate for the epigenetic regulation of neurogenesis, that has also been shown to suppress TLX expression, is the miR-9 (Zhao et al., 2009). It has been also shown to be specifically expressed in the neurogenic regions of the mouse developing and adult brain (Kapsimali et al., 2007). miR-9 has been shown to control the timing of neurogenesis in the developing zebrafish (Coolen et al., 2012) and to promote neuronal differentiation of NSCs from human (Xue et al., 2016) and mouse (Zhao et al., 2009) origin. The sequence of miR-378 is embedded within the first intron of a gene that transcriptionally regulates oxidative energy metabolism by cells (*ppargc1b*) (Eichner et al., 2010). A number of studies have implied involvement of miR-378 in wide range of biological processes including cellular metabolic pathways such as mitochondrial energy homeostasis, muscle development and modulation of angiogenic and tumour suppressor factors (reviewed by (Krist et al., 2015). As with the above-mentioned microRNAs, miR-378 plays a role in inducing NSC neuronal differentiation while suppressing NSC proliferation in a TLX-

dependent manner (Huang et al., 2015). It needs to be established in future whether these microRNAs maintain the same functions *in vivo*.

1.5.4.5 GADD45β

Growth arrest and DNA-damage-inducible protein 45 β (GADD45β) is translated from a family of genes (Gadd45) that become activated in response to stress, and participate in several biological processes such as apoptosis, senescence and the cell cycle (Hollander and Fornace, 2002). Gadd45α and Gadd45β were found to promote active DNA demethylation in Xenopus oocytes thereby causing epigenetic activation of genes important for proliferation (Barreto et al., 2007). Additionally, Gadd45β expression in the DG of adult mice was shown to increase significantly in response to activity-induced (by electroconvulsive treatment (ECT) or spatial exploration task) hippocampal neurogenesis (Ma et al., 2009). Furthermore, in adult transgenic mice lacking Gadd45β expression, ECT and spatial exploration failed to induce increase in hippocampal neurogenesis, positioning Gadd45β as an important regulator for activity-induced plasticity. Last but not least, activity-induced adult born granule cells exhibited impaired morphology as measured by dendritic length and the degree of arborization in Gadd45β^{KO} mice compared to their wildtype littermates (Ma et al., 2009). As with the Xenopus system, Gadd45β was shown to induce its effects in the adult mouse hippocampus via DNA demethylation, which promotes the expression of target genes important for adult neurogenesis such as BDNF and FGF (Faigle and Song, 2013).

1.5.4.6 TET1

Ten-eleven translocation methylcytosine dioxygenase 1 (TET1) is a member of the TET family of enzymes, and functions as catalyst for the conversion of the DNA base 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) via oxidation of 5-mC (Ito et al., 2011; Pera, 2013). The conversion of 5-mC to 5-hmC is suggested to be the first step in active DNA demethylation in mammals and has also been shown to promote the reprogramming of murine somatic cells into induced pluripotent stem cells (iPSCs) (Chen et al., 2015; Ito et al., 2011; Pera, 2013). Next to being extensively expressed throughout the brain and in the adult hippocampus in particular (Szwagierczak et al., 2010), TET1 was shown to modulate the neuronal activity-induced demethylation of BDNF and FGF in the adult mouse DG. TET1 is thus as an important regulator of activity-induced hippocampal neurogenesis (Guo et al., 2011a).

1.5.4.7 FMRP & FXR2

Fragile X mental retardation protein (FMRP or FMR1) and its autosomal paralog Fragile X mental retardation syndrome-related protein 2 (FXR2) are involved in translation of mRNAs and protein synthesis-dependent plasticity (Bassell and Warren, 2008; Faigle and Song, 2013). FMRP has also been suggested to be involved in mRNA transport in dendrites as well as axonal guidance, synaptic development and neuronal circuits (Bassell and Warren, 2008). With respect to adult neurogenesis, FMRP has been shown to affect both hippocampal and SVZ neurogenesis, while FXR2 affects hippocampal but not SVZ neurogenesis (Faigle and Song, 2013). For instance, in the absence of functional FMRP in adult NPCs neurogenesis was downregulated in both neurogenic niches of the adult mouse brain – the SVZ and SGZ – such that NSC

proliferation was increased but the fate specification balance was tipped towards astrocytic rather than neuronal differentiation. Further, the authors showed that this was as a result of FMRP-mediated translation of factors involved in NSC proliferation and differentiation such as GSK-3β and members of the Wnt signalling pathway (Luo et al., 2010). Next to replicating these data, another group of researchers illustrated that lack of functional FMRP further led to impaired hippocampal-dependent learning, suggesting a significant role of FMRP in the regulation of adult neurogenesis (Guo et al., 2011b). In contrast, FXR2 mediated hippocampal, but not SVZ, adult neurogenesis by promoting BMP signalling and reducing Noggin expression, resulting in reduced NSC proliferation and increased neuronal differentiation (Guo et al., 2011c). This implies that both proteins, albeit belonging to the same family, exercise different modes of regulation of adult neurogenesis, illustrating the complexity that epigenetic regulation entails. Combined together, the variety of epigenetic mechanisms contribute to the fundamental fine-tuning and coordinating of the expression of wide array of genes that are involved in different steps of the neurogenic process (Sun et al., 2011b).

1.5.5 Transcription factors

Transcription factors are proteins that bind to specific DNA sequences thereby controlling the rate of transcription of DNA to mRNA by turning genes on (activators) or off (repressors) (Latchman, 1997). During embryonic and early development, a fine balance of transcription factor expression has been implicated in the regulation of NPCs proliferation, neuronal differentiation and NSC fate (Englund et al., 2005;

Hodge and Hevner, 2011). Summarized below is the expression and action of several transcription factors, which are important for adult hippocampal neurogenesis.

1.5.5.1 SOX2

SOX2 is highly expressed in the neuroepithelium of the embryo as well as by NSCs and NPCs of the adult brain (Episkopou, 2005; Hutton and Pevny, 2011). In the chick and mouse embryo, SOX2 signalling was established as a necessary mechanism for maintenance of NSCs and NPCs in their proliferative non-differentiative state (Graham et al., 2003). SOX2 is one of the best studied transcription factors in relation to adult hippocampal neurogenesis and has been shown to be robustly expressed by Type 1 cells in the SGZ (Hodge and Hevner, 2011). A targeted deletion of SOX2 from NSCs of the adult neurogenic regions in the brain resulted in marked reduction of NPC proliferation, depletion of Type 1 radial glia in the DG and overall reduction in neurogenesis, highlighting the importance of SOX2 in NSC pool maintenance (Ferri et al., 2004). Furthermore, after a series of experiments where SOX2 was deleted during different periods of the life span (from embryo through adulthood), it was shown using combination with chromatin immunoprecipitation (ChIP) assay, that a lack of SOX2 during early life obstructed normal hippocampal development, while deletion of SOX2 later in life led to decrease in proliferation, neurogenesis and an overall diminished NSC pool in the mouse hippocampus. These effects were mediated through SOX2-dependent regulation of the Shh signalling pathway (Favaro et al., 2009). Furthermore, two other members of the SOX family have been shown to be prominently established in the neurogenic niche and on immature adult born neurons specifically, namely SOX3 and SOX11. However, their function of maintaining

neuronal survival and supporting the neurogenic process needs further investigation (Hodge and Hevner, 2011).

1.5.5.2 Ascl1 & NeuroD1

A number of members of the bHLH family of dimerizing transcription factors have been shown to be strongly expressed in the neurogenic niches of the adult brain and some of them have also been established as direct regulators of neurogenesis (Hodge and Hevner, 2011). For instance, Hes5 has been found on both quiescent and active NSCs in the adult SGZ (Lugert et al., 2010), while Neurogenin-2 (Neurog2) is expressed on late Type 2a and early Type 2b progenitors (Roybon et al., 2009). Further research is needed to determine the precise function and mechanism of action of Hes5 and Neurog2, hitherto gain- and loss- of function studies suggest the involvement of Neurog2 in granule neuron specification in the adult DG (Roybon et al., 2009). Mammalian achaete-scute homolog 1 (Ascl1 or Mash1), expressed on Type 1 and Type 2a cells (Kim et al., 2007), and NeuroD, expressed on Type 2b and Type 3 cells, are also members of the bHLH family. However, their function in adult hippocampal neurogenesis has been studied more extensively (Hodge and Hevner, 2011). An *in vivo* analysis of the lineage of cells expressing Ascl1 in the mouse DG over period of 6 months, showed that Ascl1 is predominantly a marker for Type 2a cells, and the Type 1 cells that begin to express this transcription factor are radial glia whose self-renewal potential is limited and are on the verge of differentiating into Type 2a cells. Thus, the authors illustrated two distinct populations of Type 1 cells: those not expressing Ascl1 (GFAP+Ascl1-) and those cells in which the transcription factor had become activated (GFAP+Ascl1+). What is more, by 6 months, all Ascl1-expressing cells in the DG

from the start of the study had adopted a granule neuronal phenotype (Kim et al., 2007). Interestingly, overexpression of Ascl1 induced an oligodendrocytic fate adoption of NS/PCs in the DG of both adult rats and mice. The increase of adult born oligodendrocytes was at the expense of adult born neuronal numbers and this effect was specific to the SGZ, as Ascl1 overexpression had no effect on neurogenesis in the SVZ of these animals (Jessberger et al., 2008). Interestingly, these findings point to the lack of restrictions of fate specification the fate plasticity of adult NSCs, and also to the heterogeneity of the neurogenic niches (Faigle and Song, 2013). The transcriptional activator NeuroD1, expressed on Type 2b and Type 3 cells in the adult SGZ, has been shown to be a target of the Wnt signalling pathway (Faigle and Song, 2013). More recently, it has been illustrated to be an important re-programmer of reactive glial cells, converting them into functional neurons in the mouse brain, post injury and in an Alzheimer's disease model *in vivo* (Guo et al., 2014). Mice with constitutive knockout of the NeuroD1 gene do not survive beyond P2 (Hodge and Hevner, 2011). However, in a conditional knockout model, where NeuroD1 was deleted specifically from adult stem cells in the mouse brain, there was a significant decrease of newborn neurons in the hippocampus and olfactory bulb of the mice. What is more, the researchers showed that rather than a deficit in NPC proliferation or altered differentiation, the reduction in neurogenesis was due to impaired survival and maturation of the adult-born granule cells (Gao et al., 2009). Additionally, when isolating neurospheres from the wildtype mice and the mice that had undergone conditional NeuroD1 deletion, the authors showed that NeuroD1 is required for neuronal but not astrocyte differentiation of the neurospheres (Gao et al., 2009). Future studies need to clarify whether a cause-and-effect relationship exists between the adult

born neuronal cell death and failure to mature in the conditional knockout mouse model.

1.5.5.3 Tbrs, Pax-6 and Prox1

T-box brain 1 (Tbr1) and Tbr2 are members of the family of transcription factors that share a common DNA-binding domain, the T-box, and are involved in the regulation of radial glia and differentiation and migration of neurons during normal brain development (Englund et al., 2005; Hevner et al., 2001; Sessa et al., 2008). Within the adult SGZ, the paired domain protein Pax-6 is a marker of Type 1 and Type 2a cells, Tbr2 is present on Type 2a and Type 2b cells and Tbr2 expression within the adult mouse DG is increased in response to voluntary wheel running (Hodge et al., 2008). Tbr1 expression is restricted to postmitotic cells and is most prominently found on newborn granule neurons (Hodge et al., 2008). Additionally, the sequential expression of these transcription factors appears to follow the lineage differentiation from NSCs expressing Pax-6 through NPCs expressing Tbr2 and finally to neuroblasts and immature neurons expressing Tbr1. As Pax6 becomes downregulated, Tbr2 expression is increased (Englund et al., 2005). While the functional role of Tbr1 and Pax-6 in the process of adult neurogenesis remains to be elucidated, a conditional Tbr2 knockout mouse model revealed that in the absence of Tbr2, NSCs are actively proliferating and expanding their pool in the SGZ but without successfully differentiating into NPCs. Further, after culturing neurospheres from the hippocampi of these mice, the authors showed that Tbr2 induces NSC differentiation and neuroblast generation by repressing SOX2 activity (Hodge et al., 2012). In addition, PROX1 is another transcription factor that regulates NPC maintenance and neuronal differentiation in the adult mouse DG

(Lavado et al., 2010). As opposed to Tbr2, PROX1 mediates its effects in a Notch-dependent signalling pathway and similarly to Tbr2, PROX1 is expressed on Type 2a and Type 2b cells in the adult SGZ (Lavado et al., 2010).

1.5.5.4 CREB

CREB is a transcriptional repressor and activator of over 4000 genes, some of which are plasticity-related genes such as BDNF, c-fos, and the nerve growth factor inducible VGF (Zhang et al., 2005). CREB signalling through cAMP is involved in the regulation of circadian rhythms (Dibner et al., 2010; Ginty et al., 1993), neuronal plasticity (Sakamoto et al., 2011), LTP (Kaleem et al., 2011) and memory formation (Yin and Tully, 1996) among others. The cAMP-CREB cascade has been established as an important regulator of adult hippocampal neurogenesis. Initially, it was shown that activation of the cascade increased the proliferation of NPCs in the adult mouse hippocampus, which ultimately resulted in increased numbers of newborn neurons four weeks post intervention (Nakagawa et al., 2002). Subsequently, activation of the cAMP-CREB cascade was illustrated to bring about increased morphological complexity of mature granule cells in the adult murine SGZ labelled through Golgi stain. Activation of the cAMP-CREB cascade did not produce the same result in mice expressing a dominant-negative mutant of the CREB protein in the hippocampus (Fujioka et al., 2004). Finally, half a decade later it was shown that CREB is active in adult born hippocampal neurons for a critical period of their development (day 5-7 after birth) and targeted loss of CREB function in these cells results in impaired dendritic development as well as decreased expression of proneuronal factors such as NeuroD and DCX. Additionally, adult born neuronal survival was markedly reduced

when CREB was inactivated during the critical period (Jagasia et al., 2009). Interestingly, impaired GABA signalling during neuronal development could be compensated for by enhanced CREB signalling (Hodge and Hevner, 2011).

To sum up, adult hippocampal neurogenesis is a complex process regulated by a multitude of extracellular and intracellular factors that interact with each other in a time- and cell-specific manner. Certain pathways run in parallel to each other, so that if one fails another can compensate, while others serve to activate or inhibit key signals for the initiation of transition from one stage of the process into the next. This balance of activity-dependent signalling is very delicate and reminiscent of a symphony orchestra (see Figure 1.6; Table 1.1). However, it remains to be seen if there is one major conductor for the neurogenic process. The transcription factor TLX has been termed a master regulator of neurogenesis, and a discussion whether TLX satisfies this condition will be the focus of the next section.

Table 1.1: Pathways regulating adult hippocampal neurogenesis

Signalling Molecule	**Intervention**	**Species**	**Role of molecule in neurogenesis**	**References**
Growth Factors				
IGF-1	Peripheral administration	Adult Rats	Proliferation & Neurogenesis ↑ Astrogenesis ↔	(Aberg et al., 2000)
	I.c.v. infusion	Aged Rats	Proliferation & Neurogenesis ↑ Differentiation ↔	(Lichtenwalner et al., 2001)
	Transgenic knockdown	Adult mice	Proliferation & Neurogenesis ↑	(Yuan et al., 2015)
	In vitro administration	Primary adult rat HPC	High dose: Proliferation ↑ Low dose: Neuronal differentiation↑	(Aberg et al., 2003)
IGF-2	*In vitro* administration	Primary adult mouse HPC	Proliferation ↑	(Bracko et al., 2012)
	In vivo knockdown	Adult mice	Proliferation ↑	
FGF2	Lentiviral selective inhibition/enhancement of FGF signalling in hippocampus	Adult and aged mice	NSC maintenance, NPC proliferation & neurogenesis ↑	(Kang and Hebert, 2015)
	i.c.v. infusion of FGF-2	Aged rats	Neurogenesis ↑; Dendritic morphology ↑	(Rai et al., 2007)
VEGF	i.c.v. infusion VEGF	Adult rats	NSC Proliferation, Neurogenesis, Gliogenesis ↑	(Jin et al., 2002)
	Antidepressant treatment	Adult rats	VEGF ↑ NPC proliferation ↑	(Warner-Schmidt and Duman, 2007)
BDNF	BDNF infusion into the hippocampus	Adult rats	Neurogenesis in GCL and hilus (ectopic cells) ↑	(Scharfman et al., 2005)

Signalling Molecule	Intervention	Species	Role of molecule in neurogenesis	References
	Conditional knockdown of BDNF receptor TrkB in hippocampal NPCs	Transgenic adult mice	Proliferation & Neurogenesis ↑	(Li et al., 2008c; Li et al., 2008d)
	Conditional knockdown of BDNF receptor TrkB in hippocampal NPCs	Transgenic adult mice	Survival & Newborn neurons morphological development and functional integration ↑	(Bergami et al., 2008)
	BDNF heterozygous knockout	Transgenic adult mice	BDNF necessary for environmental enrichment-induced increase in hippocampal neurogenesis	(Rossi et al., 2006)
NT-3	Conditional NT-3 knockdown	Adult mice	NPC Proliferation ↔ Neurogenesis ↑ Astrogenesis ↔	(Shimazu et al., 2006)
Neurotransmitters				
Dopamine	PD model	MPTP mice	Depletion of dopamine = ↓ NPC proliferation in SGZ in rodents	(Höglinger et al., 2004)
	Lesion in brain with 6-OHDA	Adult rats		
	IHC	Human brains of PD patients	PD patients = ↓ number of NPCs in SGZ	
Glutamate	Retrovirus-mediated knockout of glutamate receptor NMDAR	Adult mice	Newly born neuronal survival and functional integration in hippocampal circuitry ↑	(Tashiro et al., 2006)
GABA	Retroviral labelling of adult-born hippocampal neurons and electrophysiological recording of the cells	Adult mice	Ambient GABA tonically activates newborn granule cells in the DG, thus ↑survival	(Ge et al., 2005)

Signalling Molecule	Intervention	Species	Role of molecule in neurogenesis	References
	Viral-mediated change in $[Cl^-]_i$ in newly-born hippocampal neurons so that GABA is inhibitory		Conversion of GABA excitatory property to inhibitory in newborn granule cells in the DG results in impaired dendritic development and synapse formation	
	In vivo knockdown of $GABA_A$ subunits in the hippocampus; electrophysiology, viral delivery for labelling of different cell types; IHC	Adult mice	Tonic GABA input from parvalbumin-expressing hippocampal interneurons promotes quiescence of hippocampal Type 1 NSCs; NSC maintenance ↑	(Song et al., 2012)
5HT	Acute administration of agonists and antagonists of different 5-HT receptors; IHC outcome	Adult rats	5-HT stimulates hippocampal NPC Proliferation ↑	(Banasr et al., 2004)
	Administration of $5\text{-}HT_{1A}$ antagonist + BrdU	Adult rats	30% decrease in hippocampal NPC proliferation => 5-HT →Proliferation ↑	(Radley and Jacobs, 2002)
Ach	Immunotoxin-mediated lesion of the cholinergic neurons of the forebrain projecting to the DG; IHC	Adult rats	Survival (BrdU+NeuN+) ↓ Apoptosis	(Cooper-Kuhn et al., 2004)
	Pharmacological increase in the level and duration of action of Ach; i.p. administration; IHC	Adult mice	Neuronal survival ↑ NPC proliferation ↔	(Kaneko et al., 2006)
Noradrenaline	Neurotoxic selective depletion of Ne	Adult rats	NPC proliferation ↓ Survival ↔ Differentiation ↔	(Kulkarni et al., 2002)
	Pharmacological induction of Ne release	Adult rats	NPC proliferation ↔ Neuronal survival ↑	(Rizk et al., 2006)

Signalling Molecule	Intervention	Species	Role of molecule in neurogenesis	References
	In vitro system treated with Ne	Adult hippocampal Type 2a progenitors	Proliferation ↑	(Masuda et al., 2012)
Morphogens				
Notch homolog family	Inducible gain/loss of function of Notch1; IHC	Adolescent and adult transgenic mice	Notch1 and NICD expressed in SGZ from birth to adulthood Activated Notch1 = Proliferation ↑ Notch1 depletion or NICD depletion = cell cycle exit, NSC to NPC conversion; NCID overexpression = enhanced dendritic complexity & neuronal survival Notch1 depletion = impaired dendritic morphology	(Breunig et al., 2007)
	Conditional deletion of Jagged1; IHC	Adolescent and adult transgenic mice	Adolescence: Jagged1 knockdown = smaller DG Adulthood: Jagged1 knockdown = neurogenesis transient ↑ + NSC pool ↓	(Lavado and Oliver, 2014)
	Conditional deletion of Rbpj	Adult transgenic mice	Transient increase in neurogenesis + depletion of NSC pool	(Imayoshi et al., 2010)
Wnts proteins and the Wnt signalling pathway	Gain- and loss- of function of the canonical Wnt/β-catening pathway through lentiviral administration; IHC	Adult hippocampal NPCs Adult female rats	Both *in vivo* and *in vitro*: Activation of Wnt/β-catenin = Neurogenesis↑ (DCX+ & BrdU+/DCX+); Inhibition of Wnt/β-catenin = Neurogenesis↓	(Lie et al., 2005)
	Investigating within-niche Wnt signalling using a novel reporter	Adult rat hippocampal NPCs and human NPCs	Autocrine Wnt signalling = NSC maintenance, enhancing cell-cycle reentry and repressing differentiation	(Wexler et al., 2009)

Signalling Molecule	Intervention	Species	Role of molecule in neurogenesis	References
	system *in vitro* + Wnt antagonists & agonists Inhibit neurogenesis by lentiviral blocking of Wnt signalling in the DG	Adult male rats	Neurogenesis ↓ (DCX+ cells) coupled with impaired performance on neurogenesis-dependant behavioural tasks (MWM, NOR)	(Jessberger et al., 2009)
BMP	Lentiviral knockdown of various members of BMP signalling pathway; IHC; Noggin administration i.c.v. Transgenic mice and lentiviral gain- and loss- of function of different members of BMP signalling pathway; IHC	Adult mice; NSC hippocampal cultures Adult mice	Maintenance of NSC quiescence (non-proliferating, non-differentiating) both *in vitro* and *in vivo* and important for long-term activity of hippocampal NSCs BMP signalling regulates the tempo of NPC maturation – pushes NPCs into quiescence; Inhibition of BMP signalling = NPC activation and short-term neurogenesis↑	(Mira et al., 2010) (Bond et al., 2014)
Shh	AAV-mediated overexpression of Shh in hippocampus Gene deletion/overexpression of members of the Shh pathway; IHC	Adult rats and SGZ NPCs *in vitro* system Adult transgenic mice	Time- and doze- dependent increase in NPC proliferation both *in vivo* and *in vitro* Shh signalling and primary cilia control the formation of adult NSC pool in the DG as well as proper formation and expansion of DG during postnatal development	(Lai et al., 2003) (Han et al., 2008)

Signalling Molecule	Intervention	Species	Role of molecule in neurogenesis	References
	Cell fate mapping, IHC, Cre-mediated genetic modifications	Adult transgenic mice	qNSCs and NPCs in SGZ respond to Shh signalling by Proliferation ↑	(Ahn and Joyner, 2005)
Epigenetic factors				
MBD1	Constitutive knockout of MBD1 since embryo; IHC; behavioural test	Adult mice $MBD1^{-/-}$	Hippocampal neurogenesis ↓; spatial learning ↓; LTP in the DG ↓	(Zhao et al., 2003)
	In vitro; Western blot; ChIP assay; MBD1 knockdown; FGF2 overexpression	Brain-derived adult mouse/rat NSPCs	Mbd1 epigenetically regulates FGF2 expression to promote adult NSPC neuronal differentiation	(Li et al., 2008b)
	In vitro; Western blot; ChIP assay; retro/lentiviral assays *in vivo*	Adult NSCs isolated from $MBD1^{-/-}$ and WT male mice	MBD epigenetically regulates miR-184 & Numbl to form regulatory network that controls balance between proliferation and differentiation of NSCs	(Liu et al., 2010a)
MeCP2	*In vivo* and *in vitro* transgenic model; IHC & retroviral labelling	$MeCP2^{KO}$ & WT adolescent/adult mice;	Neurogenesis ↔; Neuronal maturation ↓; Dendritic spine density ↓; Synaptogenesis ↓	(Smrt et al., 2007)
	Lentiviral transfection (shRNA & overexpression); ChIP; IP; Western blot; Luciferase assay	Adult NSCs isolated from MeCP2 KO mice	MeCP2 & Sox2 corepress miR-137 to inhibit NSC proliferation and induce neuronal differentiation	(Szulwach et al., 2010)
HDACs	NeuroD overexpression *in vitro*; VPA administration *in vivo* and *in vitro*; IHC, PCR, Western blot;	Adult female rats and hippocampal NPC cultures	*In vitro*: HDAC inhibition = Neuronal Differentiation ↑ through activation of NeuroD; *In vivo*: HDAC inhibition = NPC proliferation ↓ & Differentiation ↑	(Hsieh et al., 2004)

Signalling Molecule	Intervention	Species	Role of molecule in neurogenesis	References
	IP; ChIP; RNA interference assays; lentiviral transfections; IHC	NSC cultures	HDACs maintain NSCs in proliferative non-differentiative state via TLX-mediated pathway	(Sun et al., 2007)
	Compare neurogenesis across life span in HDAC2-deficient mice	Adult mice and embryos	HDAC2 is required for full differentiation and survival of DG newborn neurons in adulthood but not development	(Jawerka et al., 2010)
miR-184	See notes on MBD1			(Liu et al., 2010a)
miR-137	See MeCP2 Overexpression and silencing of MiR-137; IHC; Morphological analysis	See MeCP2 Hippocampal NSC cultures; *In vivo* studies with mice	See MeCP2 miR-137 ↑ = inhibition of dendritic morphogenesis, phenotypic maturation and spine development both *in vivo* and *in vitro*	(Szulwach et al., 2010) (Smrt et al., 2010)
	ChIP; IHC; Lentiviral transfection; *In utero* electroporation	Embryonic NSC culture	Control of NSCs fate determination; inhibits proliferation and accelerates neuronal differentiation; forms regulatory loop with TLX and LSD1	(Sun et al., 2011a)
miR-219	ChIP; IHC; Lentiviral transfection; *In utero* electroporation	Murine embryonic NSCs cultures	miR-219 supresses NSC proliferation and TLX expression, but also regulated by TLX	(Murai et al., 2016)
miR-Let-7a	miR-let7a mimics and inhibitors; IHC	Primary embryonic NSCs	miR-let7a promotes NSC neuronal differentiation	(Song et al., 2015)
miR-let7b	*In Utero* electroporation & Cell cycle analysis; IHC	Primary adult NSCs	miR-let7b targets TLX and cyclin D1 to reduce NSC proliferation and increase neuronal differentiation	(Zhao et al., 2010)

Signalling Molecule	Intervention	Species	Role of molecule in neurogenesis	References
miR-Let-7d	ChIP; IHC; Lentiviral transfection; *In utero* electroporation	Primary murine NSCs	miR-let7d targets TLX/miR-9 cascade to reduce NSC proliferation and increase neuronal differentiation	(Zhao et al., 2013a)
miR-9	ChIP; IHC; Lentiviral transfection; *In utero* electroporation	Primary adult mouse NSCs	miR-9 targets TLX to reduce NSC proliferation and induce neuronal differentiation	(Zhao et al., 2009)
miR-378	Cell transfection; dual luciferase reporter assay	Primary adult mouse NSCs	miR-378 targets TLX to reduce NSC proliferation and induce neuronal differentiation	(Huang et al., 2015)
GADD45β	Knockdown (siRNA) of Gadd45β	Embryonic Xenopusoocytes	Gadd45β promotes epigenetic gene activation by repair-mediated DNA demethylation => activity-induced proliferation	(Barreto et al., 2007)
	Gadd45β knockout; IHC; PCR; Electroconvulsive treatment	Adult transgenic mice & wildtypes	Gadd45β promotes NPC proliferation, dendritic growth and maturation of adult born neurons	(Ma et al., 2009)
TET1	AAV overexpression of TET1 in the DG; PCR; DNA sequencing; ECT	Adult mice	TET1 regulates activity-induced demethylation of neurogenesis-promoters BDNF and FGF in the adult mouse DG	(Guo et al., 2011a)
FMRP	Knockdown of FMRP *in vivo* and *in vitro*; IHC; Luciferase assays; Western blots	Adult mice: $FMRP^{KO}$ & WT; Adult primary DG NPCs	$FMRP^{KO}$ = Neurogenesis ↓ *in vivo* and *in vitro*; Astrocyte differentiation ↑	(Luo et al., 2010)
	Retroviral-mediated knockdown of FMRP in aNSCs *in vivo* and *in vitro*; IHC; Behavioural testing	Adult mice; Adult primary DG NPCs	FMRP knockdown = Proliferation ↑ & Neurogenesis ↓ *in vivo* and *in vitro*;	(Guo et al., 2011b)

Signalling Molecule	Intervention	Species	Role of molecule in neurogenesis	References
			Astrocyte differentiation ↑; Impaired learning	
FXR2	Constitutive $FXR2^{KO}$ & lentiviral FXR2 selective knockdown; Luciferase assay; RNA-IP; PCR; IHC	Adult mice; Adult primary DG & SVZ NPCs	$FXR2^{KO}$ = Noggin ↑ and BMP signalling ↓ in DG but not SVZ => increased proliferation and altered cell fate specification of NPCs	(Guo et al., 2011c)
Transcription factors				
SOX2	SOX2-targeted deletion in NSCs during adulthood; IHC Embryonic and adult deletion of SOX2; ChIP; IHC	Adult mice Mice; adult primary NPCs	Depletion of Type 1 cells in DG; Proliferation ↓ Adulthood: Neurogenesis ↓ through SOX2 regulation of Shh pathway	(Ferri et al., 2004) (Favaro et al., 2009)
Ascl1	Overexpression of Ascl1; IHC	Adult mice & adult rats	Directed differentiation of hippocampal NS/PCs towards oligodendrocytes instead of neurons	(Jessberger et al., 2008)
NeuroD/NeuroD1	Inducible stem cell-specific deletion of NeuroD1; IHC	Adult mice; Neurosphere cultures from adult mice	NeuroD1 is required for the survival and maturation of adult-born granule cell neurons and for neuronal, but not astrocyte, differentiation in neurosphere cultures	(Gao et al., 2009)
Tbr2	Conditional knockout of Tbr2; IHC; ChIP; Tbr2 overexpression	Developing & adult mice; primary hippocampal cultures	Tbr2 knockout = activated NSCs, increased proliferation of NSCs, NSCs accumulation in SGZ; NSCs fail to differentiate to NPCs and granule neurons	(Hodge et al., 2012)
PROX1	Conditional knockdown of Prox1; IHC	Developing & adult mice	Prox1 is required for the maintenance of intermediate progenitors in the adult	(Lavado et al., 2010)

Signalling Molecule	Intervention	Species	Role of molecule in neurogenesis	References
			SGZ; Prox1 knockout = impaired NSC self-maintenance, via Notch signalling pathway	
CREB	Conditional transgenic and pharmacological activation of CREB; IHC	Adult mice	Activation of cAMP-CREB = increased NPC proliferation and increased neuronal survival (4 weeks later)	(Nakagawa et al., 2002)
	Conditional transgenic and pharmacological activation of CREB; IHC; Golgi staining	Adult mice; adult primary hippocampal cultures	Activation of cAMP-CREB = increased dendritic length and number of branch points in SGZ cells + the maturation of newborn neurons	(Fujioka et al., 2004)
	Retroviral-mediated CREB gain- and loss- of function; IHC	Adult mice	Loss of CREB impairs differentiation and morphological maturation of adult born DG neurons, via GABA but not NMDA pathway	(Jagasia et al., 2009)
TLX	Master regulator of NSC maintenance in proliferative and non-differentiative state; To be discussed in detail in the following section.			(Qu et al., 2009; Shi et al., 2004)

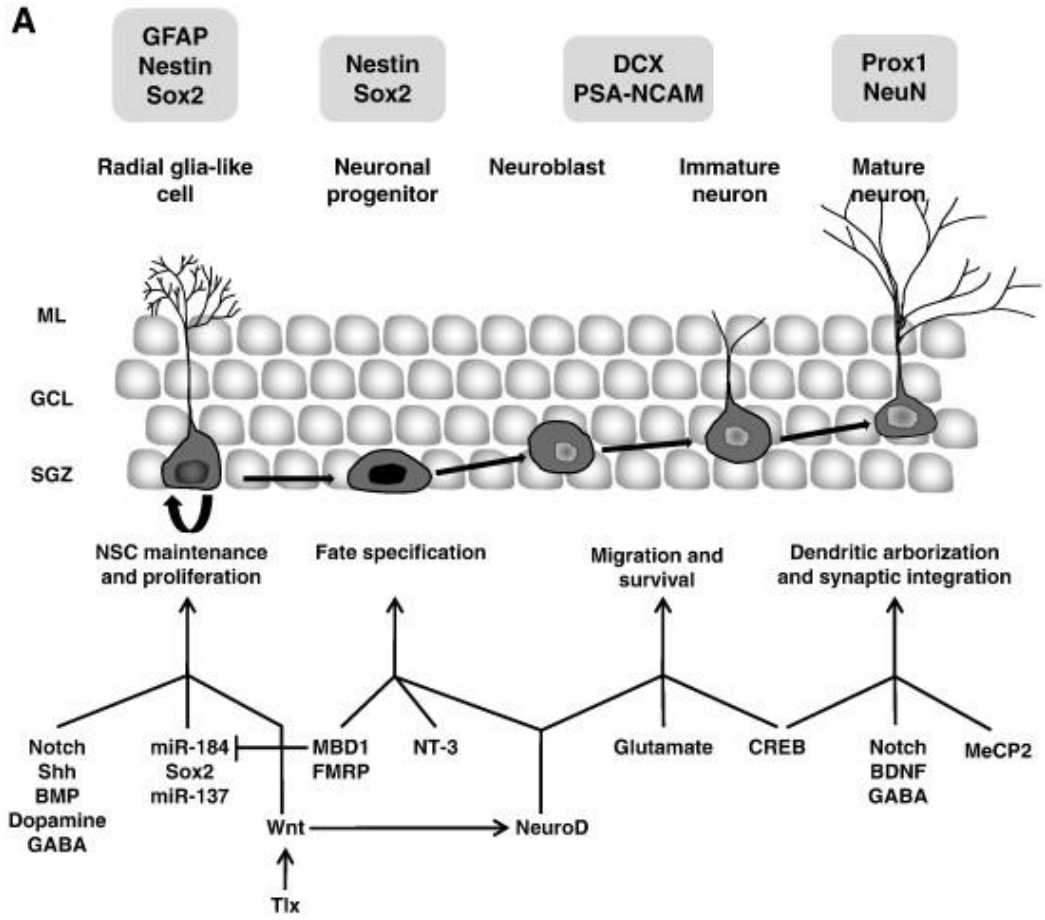

Figure 1.6: Extracellular and intracellular regulators of adult hippocampal neurogenesis. Adapted from (Faigle and Song, 2013).

1.6 TLX

Nuclear receptors are a superfamily of transcription factors, which share a similar three-domain structure (a DNA-binding domain, a ligand-binding domain and a variable N-terminal transactivation domain to which co-activators and/or co-repressors can bind) and regulate genes involved in many physiological and developmental processes (Skerrett et al., 2014). Nuclear receptors have proven to be important drug targets for a host of diseases (Sladek, 2003) and recent attention has focused on their role in neurogenesis and neurodegenerative diseases (Skerrett et al., 2014). The orphan nuclear receptor subfamily 2, group E, member 1 (NR2E1) also

known as *Tailess* or TLX, the human homologue of the *Drosophila tailless (tll)* gene has been positioned as a key regulator of embryonic and adult neurogenesis (Islam and Zhang, 2015; Monaghan et al., 1995).

1.6.1 Structure and expression of TLX

The human *TLX* gene comprises of 9 exons and encodes the TLX protein which consists of 385 amino acids (Jackson et al., 1998). It is an evolutionary conserved member of the nuclear receptor superfamily found in both vertebrates and invertebrates (Mangelsdorf et al., 1995). An alignment of the drosophila, murine and human TLX proteins reveals remarkable interspecies conservation with 70% - 99% homology between the three species (Yu et al., 1994). TLX contains the characteristic domains of the nuclear receptor superfamily, namely an N-terminal highly conserved DNA-binding domain, a flexible hinge region, followed by a moderately conserved C-terminal ligand-binding domain, but no N-terminal domain that is regulated through ligand-independent activation or repression (Benod et al., 2016). Nonetheless, the possibility that TLX can function independently of synthetic or endogenous ligand remains and is still under investigation (Sobhan and Funa, 2017). The DNA-binding domain consists of two zinc fingers which are involved in the binding to specific DNA sequences (response elements) and DNA dimerization (Gui et al., 2011). In contrast to the ligand-binding domains characteristic of nuclear receptors, the ligand-binding domain of TLX consists of 10 (as opposed to 12) α helices and a β sheet, which has been shown to result in two conformation of this C-terminal domain (Sobhan and Funa, 2017). Specifically, the missing α1 and α2 helices, along with the unique 5-amino acid insertion identified between helices α8 and α9, allow for a closed or "auto-

repressed" conformation of the TLX protein where α11 forms a kink with α10 and α12 over the ligand-binding site and prevents the binding of any ligands or co-regulators (Benod et al., 2016; Wang and Xiong, 2016). Additionally, TLX can adopt the putative canonical α-helical sandwich conformation where α12 is undocked from the ligand-binding site and can assume different positions so that the ligand-binding pocket is adaptable to different molecules (Benod et al., 2016; Sobhan and Funa, 2017).

In the mammalian organism, TLX expression is restricted to the CNS and consistent with findings from *Drosophila*, expression of TLX in the rodent is specific to the developing forebrain and retina (Monaghan et al., 1995; Yu et al., 2000; Yu et al., 1994). In the mouse embryo, TLX expression is detectable at E8, peaking at E13.5 and declining thereafter with no expression detected in the periventricular neurogenic zone after E16 and no TLX present on retinal progenitor cells after E17.5 (Monaghan et al., 1995; Yu et al., 2000). At the embryonic stage, TLX expression was found to be high at the dorsal-lateral region of the telencephalon and gradually decreased across the dorsal-ventral axis with low expression in the ventral-medial telencephalon (Stenman et al., 2003). mRNA and protein expression has been reported in the developing amygdala, striatum, hippocampus and septum as well (reviewed by (O'Leary et al., 2016b)). Postnatal TLX expression increases with high levels present in the neurogenic niches of the adult mouse brain and more specifically in the NSCs of the SGZ and the SVZ (Li et al., 2008b; Monaghan et al., 1995; Roy et al., 2004; Shi et al., 2004). TLX expression has been found in multiple organs and tissues in the human, namely the adrenal glands, the brain and cerebellum, the testis and placenta, and in

bone marrow (Jackson et al., 1998; Kumar et al., 2008; Nishimura et al., 2004). Interestingly, TLX expression was found to be increased in human glioblastomas (Liu et al., 2010b) as well as in glioma cell lines, glioma stem cell lines (Park et al., 2010b) as well as in high-grade glioma patient samples where increased level of TLX expression was correlated with poor prognosis (Parsons et al., 2008; Phillips et al., 2006; Sim et al., 2006; Zou et al., 2012). Additionally, TLX has been positioned as a potential gene of interest in relation to mental illness. Mutations of *tlx* regulatory regions as well as abnormalities at the chromosomal locus of the *tlx* gene have been associated with two heritable psychiatric conditions, namely schizophrenia and bipolar disorder (Hayden and Nurnberger, 2006; Juarez et al., 2013).

1.6.2 Function of TLX

The functional importance of TLX is now apparent from studies establishing that TLX maintains adult hippocampal neural precursor cells in a proliferative, undifferentiated state (Shi et al., 2004; Sun et al., 2007). Targeted disruption and conditional deletion in mouse models have implicated TLX as an important factor in the control and maintenance of adult hippocampal neurogenesis (reviewed by (Islam and Zhang, 2015)). Furthermore, manipulation of TLX expression in the developing mouse forebrain results in abnormal development of the amygdala, hippocampus and septum as well as impaired behavioural performance during adulthood on tasks dependent on the normal functioning of these regions (reviewed by (O'Leary et al., 2016b)). Experiments have confirmed that TLX has a role to play in spatial learning, memory and synaptic plasticity (Christie et al., 2006; Zhang et al., 2008) . Furthermore, it has been reported that TLX knockout mice demonstrate a reduction in the size of the DG

and amygdala (Monaghan et al., 1997) and exhibition of an aggressive phenotype (Young et al., 2002). These mice also demonstrate a trend for higher circulating levels of the stress hormone corticosterone and show anxiety-related behaviours (Young et al., 2002).

With respect to mice lacking TLX from utero, male and female adults of both C57BL/6J and Bl6129F1 strains exhibit increased aggression (Abrahams et al., 2005; Young et al., 2002). While the C57BL/6J males lacking TLX expression display more severe aggressive phenotype than the Bl6129F1 males lacking TLX, measured through home cage interactions and neutral arena encounters, females lacking TLX from both strains present with similarly heightened aggression within the resident-intruder paradigm and impaired maternal care evident from the premature death of their litters (Young et al., 2002). The aggressive phenotype was absent in male and female mice from both strains which were heterozygous for TLX, i.e. had one functional allele of the gene as opposed to constitutive deletion, and the females displayed normal maternal behaviour, hence rendering the heterozygotes adequate for use as breeders for transgenic studies (Young et al., 2002). Introducing the expression of the human homologue of the TLX gene in mice with constitutive deletion of TLX rescued the aggressive phenotype (Abrahams et al., 2005), suggesting a possible role for TLX in the processing of aggression in humans, however, presently no studies have examined this conjecture. Findings from the spontaneous deletion mouse model regarding the increased aggressive phenotype in the absence of TLX were replicated in adult mice where TLX expression was repressed through homologous recombination (Roy et al.,

2002), and this behavioural outcome was later shown to be mediated through serotonin receptor signalling (Juarez et al., 2013). Interestingly, suppression of TLX expression in adulthood did not result in increased aggressive behaviour (Zhang et al., 2008) indicating that TLX function during development and early life is important for the normal formation of neural pathways that regulate emotional processing.

Overexpression of TLX during adulthood enhanced learning and memory on the MWM task, while knock down of the receptor during adulthood impeded behaviour on this hippocampal-dependent task (Murai et al., 2014; Zhang et al., 2008). The behavioural outcomes of both studies were coupled with an increase (Murai et al., 2014) and a decrease (Zhang et al., 2008) in hippocampal neurogenesis, respectively. Interestingly, no change in contextual fear conditioning, another hippocampus-dependent task, was observed when TLX expression was inhibited specifically during adulthood (Zhang et al., 2008). While fear conditioning and MWM tap into spatial and context- hippocampal-dependent memory, it has been proposed that spatial memory is particularly dependent on hippocampal neurogenesis (Jessberger et al., 2009; Snyder et al., 2005), which is the process targeted by deletion of TLX during adulthood. Nonetheless, disrupting TLX expression from early life through homologous recombination did not elicit impaired learning acquisition or memory performance on the MWM task during adulthood despite a reduction in hippocampal volume (Belz et al., 2007; Drill, 2009) and a prominent deficit in behavioural flexibility as measured by reversal learning on the MWM task (Drill, 2009), which depends on prefrontal-hippocampal circuitry (Kosaki and Watanabe, 2012). Moreover

this deletion model did not exhibit impaired spontaneous alternation in the radial maze, another hippocampal-dependent behavioural test (Drill, 2009). In light of such distinct phenotypes observed after disruption of TLX expression during either development or adulthood it would be interesting to investigate whether there are critical periods through the lifespan that render TLX function as more prominent for specific types of cognition and whether aspects such as the deletion method or sex of the animal may exert an additional effect.

Spontaneous deletion of TLX from utero resulted in an anxiolytic phenotype in both male and female adult mice from the C57BL6/J strain, but not the Bl6129F1 strain, as measured by exploratory behaviour in the EPM (Young et al., 2002). However, male mice from the Bl6129F2 strain where TLX was deleted from early life through homologous recombination, displayed increased exploration of the open arms in the EPM, thus an increased anxiolytic phenotype (Roy et al., 2002). Whether an anxiolytic phenotype is present in rodents when TLX is selectively deleted during adulthood remains unknown. Nevertheless, it appears that TLX deletion from birth affects the development of key limbic structures such as the amygdala and hippocampus which contributes to impaired anxiety-related behaviour during adulthood as measured by the EPM task (reviewed by (O'Leary et al., 2016b)). Similarly, contradictory results were obtained with respect to locomotor activity from adult male Bl6129F1 and Bl6129F2, with the former (a spontaneous deletion of TLX) presenting with hyperactivity (Wong et al., 2010), while the latter (deletion through recombinant homologue) displaying the same locomotor behaviour as controls (Roy et al., 2002).

Interestingly, the striatum was shown to remain intact in adult Bl6129F2 mice (Drill, 2009) and further when TLX was deleted during adulthood alone, no locomotor deficits were observed (Zhang et al., 2008). Thus, it is possible that hyperactivity occurred as a function of spontaneous deletion of TLX but further research needs to address this hypothesis by employing further motor performance tests. To sum up, TLX is important for normal brain development, the maintenance of postnatal neurogenesis and appears to have distinct functions through the lifespan with potential roles in emotion-, anxiety-, and cognitive- related processes as well as hyperactivity (O'Leary et al., 2016b). Future studies need to focus on delineating the effects of TLX deletion based on the time and brain area of intervention.

1.6.3 Regulators and targets of TLX

A detailed outline of the regulators and targets of TLX is presented in Table 1.2 and Figure 1.7 (see Abbreviations section for full gene and protein names used throughout this section). Under physiological conditions, TLX regulators have been shown to act on the post-transcriptional level, repressing both genetic and protein expression in order to maintain a balance between the rate of self-renewal of NSCs and the rate of neurogenesis. This means that most molecules act as accelerators of neuronal differentiation derived from the reduced symmetric and increased asymmetric division of NSCs (Rajman and Schratt, 2017). Examples of TLX regulators are miR-9 (Zhao et al., 2009) and miR-137 (Sun et al., 2011a), with which TLX forms a feedback loop to maintain the balance between NSC proliferation and differentiation. Others include miR-378 (Huang et al., 2015) and members of the miR-let7 family (Zhao et al., 2010; Zhao et al., 2013a). Under pathological conditions *in vitro* it has been shown that TLX

is downregulated in a dose- and time-dependent manner by the inflammatory cytokine interleukin-1β (IL-1β) resulting in a decrease in neurogenesis (Green and Nolan, 2012b; Green et al., 2012; Ryan et al., 2013).

During embryonic development TLX targets GSH2, a protein involved in the dorso-ventral patterning of the mammalian telencephalon. This interaction has been shown to result in correct establishment of the pallio-subpallial boundary in the embryonic mouse telencephalon (Stenman et al., 2003). Under physiological conditions during postnatal neurogenesis, TLX has been suggested to prime NSCs towards neuronal fate and differentiation by activating the pro-neurogenic factor *Mash1* (Elmi et al., 2010) and to further enhance neurogenesis through *Sirt1* activation (Iwahara et al., 2009; Wu et al., 2015). The primary role of TLX, nonetheless, is maintaining the NSC pool in proliferative and non-differentiative state and to achieve this, TLX binds to the promoters of several genes to either transcriptionally activate or repress them (Islam and Zhang, 2015). Both *pten* and *p21* have been shown to maintain stem cell quiescence in different regions of the brain and body by inhibiting cell cycle progression albeit through different pathways; the former induces apoptosis and cell cycle arrest, while the latter inhibits cell cycle progression (Cheng et al., 2000; Hill and Wu, 2009; Kippin et al., 2005; Leslie and Downes, 2004). It has been demonstrated that TLX represses the expression of both genes to maintain the NSC pool in the hippocampus (Islam and Zhang, 2015; Sun et al., 2007). Additionally, TLX maintains the hippocampal NSC pool by binding to the promoter of *Wnt7α* and thereby transcriptionally activating it (Qu et al., 2009). The retinal NSC pool on the other hand,

has been shown to be maintained through the interaction between TLX and the promoters of *p57, Plce1* and *Pax2,* where TLX acts as a transcriptional repressor of these genes, resulting in increased proliferation and reduced differentiation of neural retinal progenitors (Sehgal et al., 2009; Yu et al., 2000; Zhang et al., 2006). Additional evidence illustrating the role of TLX in NSC maintenance stems from the fact that TLX represses astrocyte differentiation in favour of NSC proliferation through interaction with the promoters of the pro-astrocytic genes *S100β, Bmp4, Aqp4* and *Gfap* (Qin et al., 2014; Shi et al., 2004). Under pathological conditions, however, TLX has been shown to activate MMP2 and interact with the tumour suppressor VHL to promote the self-renewal of NSCs in tumour formations in the brain, and in neurospheres in neuroblastoma cell lines (Chavali et al., 2014; Zeng et al., 2012). On the contrary, under hypoxic conditions TLX has a protective effect whereby it promotes NSC self-maintenance by activating the *Oct3/4* gene (Chavali et al., 2011). TLX regulates the genes outlined above by forming a transcriptional complex in a region- and cell-specific manner through the different developmental stages with either of its coregulators, LSD1, members of the HDAC family, Atrophins or BCL11A (Estruch et al., 2012; Haecker et al., 2007; Sun et al., 2010; Wang et al., 2006; Yokoyama et al., 2008; Zhang et al., 2006).

The interaction between TLX and SOX2 is different, yet. Briefly, the transcriptional regulation of NPCs by SOX2 and TLX is autonomous such that both are independently necessary for the potentiation of cell proliferation and the repression of cell differentiation in order to maintain the undifferentiated and self-renewable state of

progenitors within the neurogenic niches (Pevny and Nicolis, 2010; Shi et al., 2008). Interestingly, it has also been shown that SOX2 is involved in a negative feedback system which regulates TLX expression. Specifically, while SOX2 activates TLX transcription, TLX can suppress its own endogenous mRNA production by binding to and repressing its own promoter (Zhi et al., 2015). SOX2 can further counteract such repression in a dose-dependent manner (Shimozaki et al., 2012). Remarkably, the results from a genome-wide association study (GWAS) of transgenic mice suggested the Notch/RBPJ signalling pathway as a potential modulator of TLX activation. It thus contributes as yet another possible pathway through which TLX performs its activity as transcriptional repressor in order to ensure the NSC pool is maintained in proliferative and non-differentiative state (Li et al., 2012b).

To sum up, TLX is involved in multiple pathways through which it functions as a master regulator of neurogenesis (Islam and Zhang, 2015). However, future studies need to interrogate further the precise roles and regulatory pathways through which TLX exerts its functions *in vivo* i.e. translate the findings from molecules to behaviour.

Table 1.2: Regulators and targets of TLX

Gene/molecule name	Mode of interaction	Result of interaction	Method of discovery	Reference
NSC proliferation & neuronal differentiation				
miR-9	miR-9 represses TLX expression by targeting its 3' UTR, but TLX can also repress expression of the pri-miRNA	miR-9 suppression of TLX results in decrease in NSCs proliferation and accelerated neural differentiation	Primary NSC cultures from mouse	(Zhao et al., 2009)
miR-137	miR-137 targets LSD1, however, can be repressed by TLX	Suppression of LSD1 and thereby TLX results in decreased cell proliferation and accelerated neural differentiation	Embryonic NSCs	(Sun et al., 2011a)
miR-378	miR-378 represses TLX mRNA and protein expression	Decrease in NSCs proliferation and accelerated neuronal differentiation	Primary mouse hippocampal cultures and Luciferase assay	(Huang et al., 2015)
miR let-7b	miR let-7b represses TLX expression	Decrease in NSCs proliferation and accelerated neuronal differentiation	NSC primary culture; *in utero* electroporation	(Zhao et al., 2010)
miR let-7d	miR let-7d represses TLX mRNA and protein expression	Decrease in NSCs proliferation and accelerated neuronal differentiation	Primary mouse hippocampal cultures and Luciferase assay	(Zhao et al., 2013a)
Neurogenesis downregulation				
IL-1β	Represses TLX expression through IL-1R1	Decrease in neurogenesis	Embryonic and adult hippocampal primary cultures	(Green and Nolan, 2012b; Ryan et al., 2013)
Embryonic development				
Gsh2	Potential target of TLX (*Gsh2* expression would be repressed)	Correct establishment of the pallio-subpallial boundary in the embryonic mouse telencephalon	No causative link: transgenic mice, IHC	(Stenman et al., 2003)
Neuroblastomas				

Gene/molecule name	Mode of interaction	Result of interaction	Method of discovery	Reference
MMP-2	TLX binds the promoter and activates the expression of *MMP-2*	Promoting self-renewal of tumour spheres in neuroblastoma; correlation with poor patient survival	Tumour cell lines; ChIP & Luciferase assays	(Chavali et al., 2014)
VHL	TLX and VHL can directly bind to each other and counterbalance/stabilize each other in condition of hypoxia for instance	Counterbalance between TLX and VHL determines important fate decisions such as self-renewal and differentiation, as well as angiogenesis and anti-angiogenesis	Neuroblastoma cell lines; primate kidney epithelial cell line; ChIP assay	(Zeng et al., 2012)
Neurogenesis upregulation				
SIRT1	TLX enhances the expression of Sirt1 through binding to the TLX-activating element in the Sirt1 promoter	Transactivation of Sirt1 by TLX enhances and facilitates neurogenesis	IHC in mouse brain, mouse embryo, HEK293 cell line; prostate cancer model	(Iwahara et al., 2009; Wu et al., 2015)
Maintenance of the NSC pool				
SOX2	SOX2 binds to and upregulates TLX expression; SOX2 also interferes with TLX-mediated self-repression on its own promoter	Maintenance of stem cell self-renewal	SGZ & SVZ primary NSC cultures; Luciferase & ChIP assays	(Shimozaki et al., 2012)
Wnt7α	TLX is a transcriptional activator of *Wnt7α* and it binds directly to its promoter	Increased NSC proliferation; maintenance of the stem cell pool	Primary cultures from adult mouse hippocampal NSCs; ChIP; *in situ* hybridization	(Qu et al., 2010)
pten	TLX represses this gene	TLX represses the function of this tumour suppressor gene to induce apoptosis and cell cycle arrest (G0:G1 phase)	In cell lines, retinal progenitors, SVZ; NSCs	(Liu et al., 2008; Sun et al., 2007; Yokoyama et al., 2008; Zhang et al., 2006)

Gene/molecule name	Mode of interaction	Result of interaction	Method of discovery	Reference
p21	TLX bind its promoter and represses *p21*	TLX inhibits a *p21*-induced deficiency in cell cycle progression	Transgenic TLX knockout mice (embryo and adult), primary hippocampal cultures	(Li et al., 2008b; Niu et al., 2011; Sun et al., 2007)
p57	Potential target for repression by TLX	TLX may inhibit its (*p57*) role as negative retinal progenitor cell regulator	Embryonic and adult TLX knockout mice	(Zhang et al., 2006)
Plce1	TLX binds to its promoter and represses its expression in embryonic retina	Inhibition of Plce1 leads to increased proliferation and reduced differentiation of RPCs	TLX knockout mice; primary cultures from retinal cells; gel shift assays	(Zhang et al., 2006)
Pax2	Robust evidence for TLX repression of Pax2 by binding to its promoter site; however, some evidence that Pax2 can repress TLX	Pax2 is required for the establishment and maintenance of proper optic stalk connections and appropriate retinal morphology; balance between Pax2 and TLX is required for proper maintenance of optic nerve function; in embryo Pax2 is important for astrocyte determination and differentiation; however, overexpression of Pax2 results in similar damage as in loss of Pax2 function	TLX knockout mice; *in vivo* electroporation; primary cultures; electrophoretic mobility shift assay; ChIP	(Sehgal et al., 2009; Yu et al., 2000)
Oct3/4 (Pou5f)	TLX is a transcriptional activator of this gene under condition of hypoxia	NPCs proliferation and pluripotency	Primary NSCs; ChIP & Luciferase assays	(Chavali et al., 2011)
Notch/RBPJ	This pathway targeting TLX promotes its expression	Promoting self-renewal and proliferation of NSCs but not differentiation	Transgenic mice, GWAS	(Li et al., 2012b)
Astrocyte differentiation				
S100β	TLX binds to the promoter region of the gene to repress its expression	TLX maintains NSC pool and reduces differentiation to astrocytes	IHC in hippocampus and primary cell cultures	(Qin et al., 2014; Shi et al., 2004)

Gene/molecule name	Mode of interaction	Result of interaction	Method of discovery	Reference
Bmp4	TLX binds to the enhancer region of BMP4 to repress its expression	TLX controls the timing of the postnatal genesis of astrocytes	NSC culture from $TLX^{LacZ/+}$ mice; *in situ* hybridization; ChIP	(Qin et al., 2014)
Aqp4 *Gfap*	TLX binds to the promoter region of these gene to repress their expression	TLX maintains NSC pool and reduces differentiation to astrocytes	Primary culture of mouse NSCs	(Shi et al., 2004)
Coregulators				
Atrophins HDACs LSD1	Cofactors of TLX – association, co-interaction	These factors form a transcriptional complex with TLX in specific cells at certain developmental stages to regulate expression of target genes; mostly to facilitate NSC proliferation by repressing other genes regulating proliferation	Genetic and biochemical approaches on Drosophila embryo and TLX knockout mice (retinal cells, NSCs)	(Haecker et al., 2007; Sun et al., 2010; Wang et al., 2006; Yokoyama et al., 2008; Zhang et al., 2006)
BCL11A	Co-regulation	BCL11A (oncoprotein) potentiates the transrepressive function of TLX on gene regulation in the brain	Y2H screen followed by ChIP & in vitro reporter gene assay	(Estruch et al., 2012)

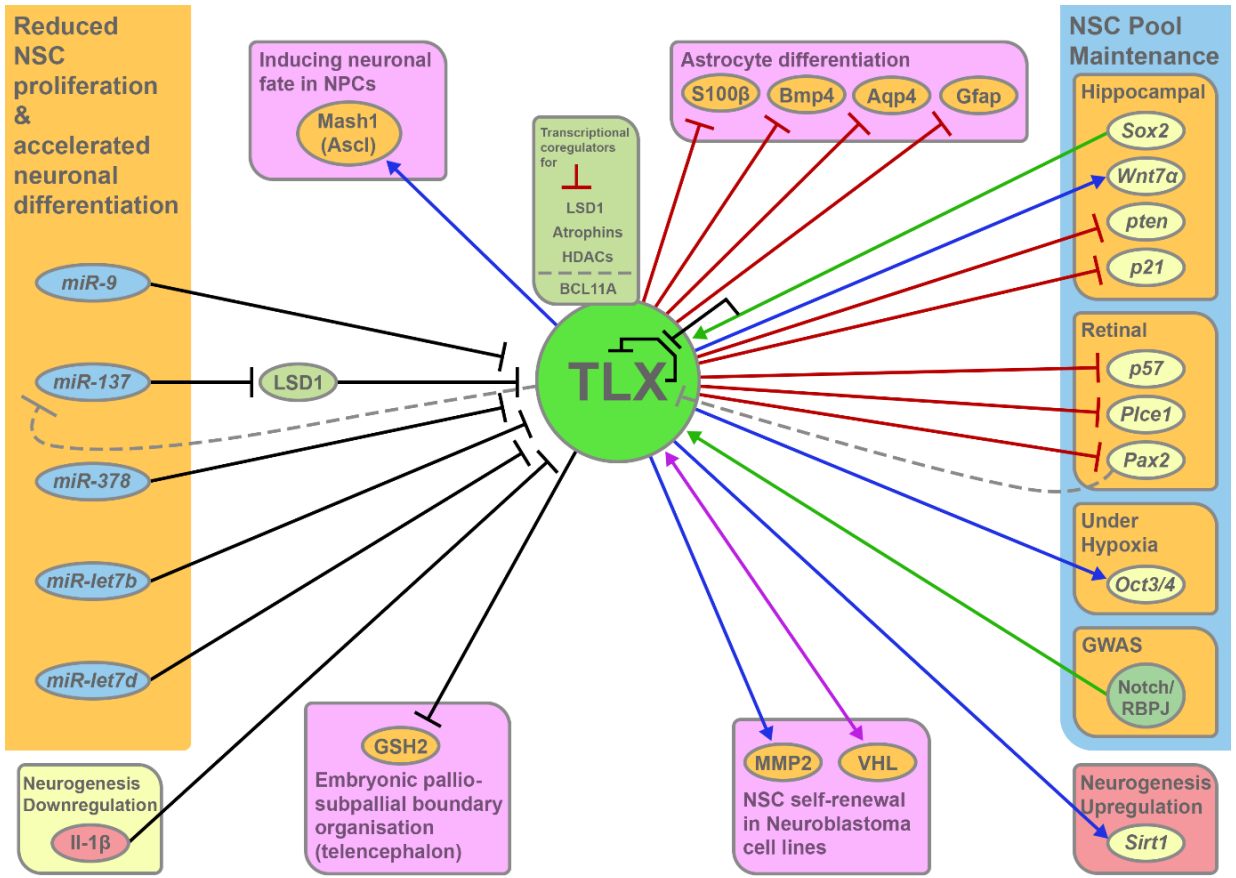

Figure 1.7: Regulators and targets of TLX. Black lines: TLX inhibitors; red arrows: target genes that TLX represses; blue arrows: gene targets TLX activates; green arrows: genes that activate TLX; purple arrows: bidirectional interactions; blue bubbles: molecules that inhibit NSC proliferation via TLX; yellow bubbles: molecules that TLX interacts with to maintain NSC pool and increase neurogenesis; red bubbles: inhibits TLX and neurogenesis; orange bubbles: other, i.e. astrocyte differentiation, maintenance of glioblastoma stem cells, embryonic stem cells,; green bubbles: coregulators.

1.6.4 Methods to parse TLX function

The TLX knockout mice can be a useful model for investigating the cognitive impairment associated with deregulation of hippocampal neurogenesis. In 2002, Simpson and colleagues discovered a novel mouse mutation (Nr2e1-null; TLX knockout), named “fierce” to reflect their aggressive phenotype, and have shown that these mice have altered neurogenesis, cortical and limbic abnormalities, aggression and cognitive impairment (Christie et al., 2006; Young et al., 2002). In this mouse model, a spontaneous deletion of all 9 exons of the TLX gene occurs *in utero* and is coupled with abnormal development and reduced neurogenesis (Young et al., 2002). Around the same time, another deletion model was developed in two other labs, such that homologous recombination was utilized resulting in a targeted deletion of TLX by disruption of exons two and three (Monaghan et al., 1997) or exons three, four and five (Yu et al., 2000) both of which occurred from embryo. Another mouse model, where TLX was deleted specifically during adulthood was created through a floxed conditional deletion where exon two was flanked by two loxP sites. Upon a transfection with a Cre-expressing virus the second TLX allele was deleted and the effects examined later in adulthood (Zhang et al., 2008). The spontaneous deletion and homologous recombination models have been examined across different mouse strains, while the Cre-induced deletion has been validated only on the C57BL6J mouse strain (Monaghan et al., 1997; Young et al., 2002; Yu et al., 2000; Zhang et al., 2008). As discussed, behavioural results have differed across mouse strains and deletion models (reviewed by (O'Leary et al., 2016b)). In order to address such discrepancies, it would be important to investigate the effects of TLX deletion in a specific brain area

such as the dDG or vDG at a specific time point of development and/or in adult life. It would also be important to utilize a large battery of tests which can clearly segregate distinct behavioural phenotypes. Due to the large amount of abnormalities that occur in the absence of TLX from birth, it is difficult to reconcile data that may appear contradictory but may be a function of confounding factors such as strain differences or deletion models. Finally, we and others have investigated the function and mechanisms of action of TLX in *in vitro* models of primary neurospheres, where it has been shown that the receptor is expressed abundantly (Green and Nolan, 2012b; Shi et al., 2004). Combining *in vitro* and *in vivo* models in different species would provide a powerful tool to test hypothesis already emerging with respect to the functional significance and therapeutic potential of the orphan nuclear receptor TLX. The regulation of hippocampal neurogenesis, however, is also orchestrated between an interplay of intrinsic and extrinsic factors that interact, inhibit and enhance one another.

1.7 Extrinsic factors regulating hippocampal neurogenesis

Extrinsic factors such as the vasculature, changes in the electrical and chemical environment, and glial immune cells control the microenvironment of the subgranular neurogenic niche (Kohman and Rhodes, 2013; Ryan and Nolan, 2016). Additionally, exercise, inflammation, ageing and diet also can modulate the rate of hippocampal neurogenesis (Hueston et al., 2017; Song, 2008). For the purpose of the present work, a positive (enrichment) and a negative (stress) extrinsic regulator of hippocampal

neurogenesis will be the focus of the current section. This is followed by a discussion on neuroinflammation as a regulator of neurogenesis.

1.7.1 Enrichment

By definition, an enriched environment is one that stimulates sensory, motor and social processing in the brain through a multitude of physical and social diversity in the surroundings (Aimone et al., 2014). With respect to rodents, an enriched environment entails housing animals with an abundance of space, toys, tunnels, running wheels, and/or other animals. Different researchers employ a collection of or single one of the above-mentioned stimuli (Olsson and Dahlborn, 2002; Rosenzweig et al., 1962). The first rodent studies examining the effects of enriched environment on plasticity reported that neuroplasticity, dendritic complexity and connectivity as well as cortical growth were enhanced upon exposure to environmental stimulation (Diamond et al., 1964; Greenough and Volkmar, 1973; Volkmar and Greenough, 1972). The pioneering study examining the effects of environmental enrichment on hippocampal neurogenesis was conducted in the lab of Fred Gage, where Kempermann and colleagues showed that four weeks after BrdU injection, animals in an enriched environment had a significantly higher number of surviving new cells which correlated with increased number of granule cells in the DG (Kempermann et al., 1997b). More recent data has shown that after living in an enriched environment for 6 weeks, mice display an increase in cell proliferation in the SGZ as measured by Ki67 immunopositivity (Karelina et al., 2012). The functional significance of this increase in granule cells in response to an enriched environment has been proposed to be improved spatial learning and memory as reflected by performance on the MWM task

(Kempermann et al., 1997b; Kempermann et al., 1998). Although the precise mechanisms underlying the effect of an enriched environment on hippocampal neurogenesis remain to be elucidated, some evidence suggests that the birth of new neurons is stimulated in an enriched environment in part by enhanced production of growth and neurotrophic factors such as VEGF and BDNF (Aimone et al., 2014). For instance, multiple reports have emerged describing the association between different types of environmental enrichment and increased hippocampal neurogenesis and BDNF levels in several strains of mice (Gualtieri et al., 2017; Jha et al., 2016; Kobilo et al., 2011) with BDNF hypothesized as the causative link between enrichment and neurogenesis in a number of studies (Bekinschtein et al., 2011; Karelina et al., 2012). Furthermore, the pro-neurogenic effects of an enriched environment in rats were inhibited when hippocampal VEGF was knocked down through shRNA-mediated interference (Cao et al., 2004). However, this finding was not replicated in a mouse model of enriched environment (Kuzumaki et al., 2011), suggesting the existence of interspecies differences in the mechanisms through which exposure to a stimulating environment may exert its effects on hippocampal neurogenesis. Nonetheless, it is important to note that the specific type of enrichment may have influenced the mechanism underlying the increase in neurogenesis not only at the molecular level, but also in terms of the stage of the neurogenic process that is being affected, i.e. proliferation, differentiation, maturation or survival. For instance, voluntary exercise or the presence of a running wheel in the cage, has been shown to be the strongest stimulus of environmental enrichment that increases the number of newborn granule cells in the hippocampus (van Praag et al., 1999a; van Praag et al., 1999b). What is more, it has been demonstrated that rodents undergoing voluntary running displayed

increased levels of growth factors coupled with improved performance on hippocampal-dependent tasks such as the MWM and enhanced numbers of proliferating hippocampal NPCs (Creer et al., 2010; Kobilo et al., 2011; Marlatt et al., 2012; van Praag, 2008; van Praag et al., 1999a; van Praag et al., 1999b). Even though some argue that running is the sole factor driving the increase in hippocampal neurogenesis observed in response to an enriched environment (Kobilo et al., 2011; van Praag, 2008), evidence from others maintain that the effects of running and other types of enrichment are additive such that voluntary running induces an increased rate of NPC cell proliferation while environmental enrichment promotes the survival of the newborn neurons (Fabel et al., 2009; Kempermann et al., 2010). Interestingly, voluntary running rescued the age-related deficits observed in hippocampal neurogenesis and newborn neuronal morphology as well as the learning and retention impairments in the spatial memory MWM task in mice. In this study levels of neurogenesis in the hippocampus of the exercising aged mice were restored to about 50% of those observed in young controls (van Praag et al., 2005). Similar to the mechanism proposed to underlie environmental enrichment-driven increase in hippocampal neurogenesis, voluntary running in rats has been shown to be strongly associated with increased expression of BDNF and other neurotrophic factors (Gomez-Pinilla et al., 2008; Neeper et al., 1996). Studies employing either blockade of BDNF production and signalling or i.c.v. administration of BDNF have elegantly demonstrated that exercise-mediated increase in hippocampal neurogenesis and improved performance on spatial learning and memory tasks such as MWM and novel object location were dependent on a BDNF-related mechanism (Gomez-Pinilla et al., 2008; Griffin et al., 2009).

Another factor that enhances hippocampal neurogenesis and is a form of cognitive enrichment is the process of learning (Zhao et al., 2008). Moreover, newborn neurons have been shown as a necessary factor for learning to occur (Shors et al., 2012). To add to the complexity of this process, a reciprocal relationship has been reported to show that increased numbers of BrdU+ cells in the rat DG were observed after learning a hippocampal-dependent task in the MWM had taken place (Ambrogini et al., 2000; Gould et al., 1999a), but not after training on a hippocampal-independent task such as delayed eye blink conditioning or cued MWM training (Gould et al., 1999a). Additionally, interfering with the learning process on the MWM task by introducing sleep deprivation or prenatal stress blocked the learning-induced increase in hippocampal neurogenesis observed in control rats (Hairston et al., 2005; Lemaire et al., 2000). Whether the interventions decreased neurogenesis first which resulted in impaired learning, or vice versa remains unclear. Interestingly, some researchers did not detect an increase in neurogenesis or in cell survival in rats that had undergone MWM training (Snyder et al., 2005; Van der Borght et al., 2005), though an increase in the number of PSA-NCAM+ cells was observed in the GCL of trained rats compared to controls (Van der Borght et al., 2005). It has been suggested that differences in the strain and age of rats as well as variations in the MWM training protocol and timing of BrdU administration may have accounted for the discrepancies (Leuner et al., 2006; Snyder et al., 2005). Furthermore, difficulties in understanding the complex interaction between learning and hippocampal neurogenesis arise from the fact that different stages of the learning process may influence distinct stages of the neurogenic process (reviewed by (Leuner et al., 2006)). For instance, when the

MWM training paradigm was disseminated into two phases, namely the rapid acquisition during the early phase of training and the late phase when improved learning is less obvious and rats reach their most optimal performance levels, the researchers found that there was no increase in NPC proliferation during the early phase of learning. However, the late phase of learning was associated with increased cell proliferation during this particular phase and decreased survival of cells generated during the early phase (Dobrossy et al., 2003). Another beautifully designed study further illuminated how not just learning per se but also the rate of acquiring new information and the difficulty of the task plays a crucial role on the outcome of hippocampal neurogenesis. The researchers used pharmacological interventions to either block or facilitate the learning processes on the classical eye-blink conditioning in rats. They found that learning an easy task resulted in the survival of relatively few adult-born neurons, while in contrast acquiring a difficult task resulted in a large number of surviving newborn cells. What is more, animals that took longer to learn the difficult task produced more surviving neurons than those who acquired the conditioning rapidly (Curlik and Shors, 2011). Thus, it is clear that a relationship between learning and hippocampal neurogenesis exists, however, the complexity of it warrants further investigation into the dynamic physiological mechanisms and processes that underlie and result from it.

1.7.2 Stress

The effects of stress on hippocampal neurogenesis have been examined at length (reviewed by (Mirescu and Gould, 2006)) and even though most evidence demonstrates a deleterious effect of stress on adult hippocampal neurogenesis

(reviewed by (Veena et al., 2011)), it has also been suggested that low levels of stress may facilitate neurogenesis (reviewed by (Saaltink and Vreugdenhil, 2014)). Various studies examining the relationship between stress and adult hippocampal neurogenesis are discussed here. For a summary of findings see Table 1.3.

Adult rats exposed to predatory odour of a fox, but not other non-threatening odours, exhibited a transient decrease in the number of proliferating NPCs that lasted up to 3 weeks after the presentation of the threat (Tanapat et al., 2001). This effect was mediated through an increase in the circulating levels of the stress hormone corticosterone (Tanapat et al., 2001). Adult tree shrews also exhibited a rapid decrease in the number of proliferating NPCs after an acute (Gould et al., 1997) or chronic (Czeh et al., 2001) exposure to a psychosocial stressor. Further evidence for the concomitant effects of stress across species on the proliferation of hippocampal NPCs and adult hippocampal neurogenesis has been shown in mice that underwent 7 weeks of chronic mild stress (Alonso et al., 2004) and marmoset monkeys exposed to an acute resident-intruder stress paradigm (Gould et al., 1998). Early life stress, such as prenatal stress and maternal separation in rats, has also been shown to have detrimental long-term effects on adult hippocampal neurogenesis, cell proliferation and performance on hippocampal-dependent tasks (Lemaire et al., 2000; Mirescu et al., 2004). Furthermore, rats subjected to chronic rather than acute psychosocial stress had a reduction in not only NPC proliferation but also in the survival of adult born granule cells (Czeh et al., 2002). Interestingly, acute restraint stress in rats had no effect on the proliferation of NPCs or the number of PSA-NCAM+ cells (newborn neurons). In

another study, chronic restraint stress for 6 weeks reduced the number of proliferating NPCs, the volume of the GCL, the number of surviving granule cells as well as the number of PSA-NCAM+ cells (Pham et al., 2003). Of interest is the fact that after 3 weeks of chronic restraint stress, rats exhibited a decrease in proliferation of NPCs but an increase in the number of PSA-NCAM+ cells, which was diminished after another 3 weeks of stress (Pham et al., 2003) suggesting that chronic stress induced a transient increase in plasticity that was not maintained when stress persisted. In line with these data, others also found a decrease in Ki67+ cells in the DG of adult rats that underwent a two week intermittent restraint stress protocol, but no change after an acute 3-hour exposure to restraint stress (Rosenbrock et al., 2005). Additionally, daily restraint stress for the period of 1 week brought about a decrease in adult born granule cells 1 and 3 weeks after cessation of the stress protocol (Luo et al., 2005). Another group of researchers found that after 3 weeks of chronic restraint stress, adult rats presented with a 70% decrease in the number of proliferating cells in the DG as well as a significant reduction in hippocampal volume compared to controls (Veena et al., 2009a). In a separate experiment the same group found that chronic restrained stress also led to a decrease in the number of surviving neurons as well as a decrease in the number of proliferating NPCs differentiating into neurons (Veena et al., 2009b). All these deficits were reversed after animals were exposed to an enriched environment for 10 days after the chronic stress was terminated (Veena et al., 2009a; Veena et al., 2009b).

Foot shocks, administered in the learned helplessness model of depression, which are a form of inescapable acute stressor decreased the proliferation of hippocampal NPCs in adult rats (Malberg and Duman, 2003; Vollmayr et al., 2003), but did not correlate with changes in the level of corticosterone in the blood (Malberg and Duman, 2003). This contrasts with the classical adrenalectomy studies that showed a suppression of the birth of both glia and neurons in the adult rat DG as well as a control over the rate of neurogenesis were mediated through adrenal hormones including corticosterone (Cameron and Gould, 1994; Gould et al., 1992). Additionally, chronic mild stress led to increased corticosterone secretion coupled with decreased hippocampal but not SVZ cell proliferation and differentiation as well as an increase in apoptosis in the DG of adult rats (Silva et al., 2008).

The relationship between the effect of stress on adult neurogenesis and circulating corticosterone levels has been complex and paradoxical to understand (reviewed by (Schoenfeld and Gould, 2012; Schoenfeld and Gould, 2013)). What is more, social isolation which is a mild stressor for mice, delayed the positive effects of running on adult neurogenesis, and resulted in elevated corticosterone levels in response to stress in individually housed runners compared to group housed mice undergoing exercise. Notably, all running mice presented with increased corticosterone levels during their active phase (Stranahan et al., 2006). Additional evidence for the positive effects of acute but not chronic stress on hippocampal neurogenesis, came from a study illustrating that 3-hour immobility stress, but not foot shock or exposure to a novel environment, resulted in an increase in proliferating NPCs in the DG of adult rats,

which was coupled with increased levels of corticosterone. Foot shock stress and novel environment stress had no effect on plasma corticosterone levels (Kirby et al., 2013). The model proposed by Saaltink and Vreugdenhil reconciles some of these discrepancies. They suggest that the relationship between neurogenesis and corticosterone, as measured by the activation of its respective glucocorticoid receptors (GRs), resembles an inverted U-shaped curve such that low levels of stress such as impoverished environment or single housing induce low levels of GR activation and NPC proliferation and survival, while controllable or escapable stress caused by physical activity and exposure to a novel enriched environment coincides with increased activation of GRs and increased NPCs proliferation and newborn neuronal survival. Finally, too much GR activation caused by uncontrollable stress such as chronic restraint, social defeat or foot shock is associated with a decrease in proliferation and neuronal maturation (reviewed by (Saaltink and Vreugdenhil, 2014); see Figure 1.8).

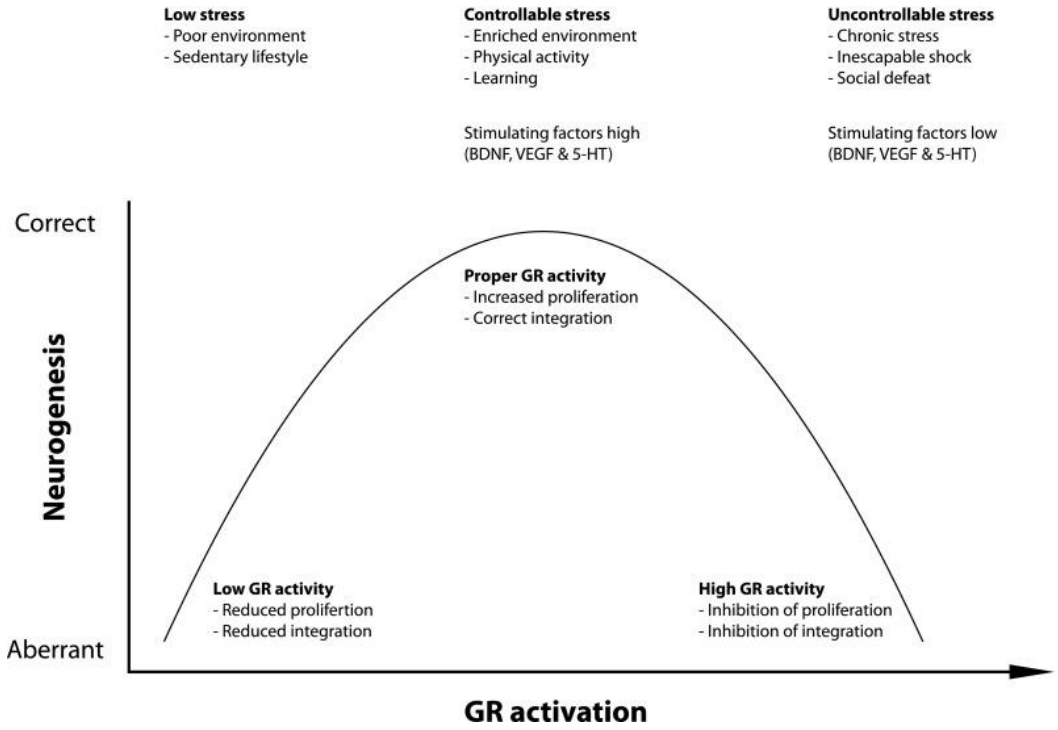

Figure 1.8: Relationship between GR activation and hippocampal neurogenesis. Adapted from (Saaltink and Vreugdenhil, 2014).

Support for this model comes from studies showing that different stressors resulted in different stages of the neurogenic process being affected (Saaltink and Vreugdenhil, 2014). For instance, chronic mild stress which was employed in the form of various daily stressors such as brief restraint, housing in impoverished environment, restricted access to food and water, or changes in the pattern of light-dark cycle, did not affect proliferation of NPCs in the adult rat DG but negatively affected the survival of adult born granule cells (Lee et al., 2006). Furthermore, acute psychosocial stress also led to a decrease in short- and long-term neuronal survival of adult born granule cells without changing the NPC proliferation rate immediately after experience of the stressor (Thomas et al., 2007). Interestingly, in another paradigm of chronic mild stress where animals were subjected to unpredictable stressors daily for 24 days, NPC proliferation and apoptosis of newborn cells were suppressed. The same study also examined the effects of acute stress and found that proliferation of hippocampal NPCs

was again decreased, while apoptosis was increased (Heine et al., 2004b). Importantly, the authors found that animals that underwent acute stress showed recovery in proliferation rates 24 hours later, and that chronically stressed rats showed recovery, albeit still not reaching proliferation rates observed in control animals, 3 weeks after cessation of the stress protocol (Heine et al., 2004b). Additionally, in a social defeat stress study in adult mice where neurogenesis was ablated in half of the animals through cranial irradiation, the authors found that the time window after termination of the chronic stress protocol represented a critical period where neurogenesis could be affected either negatively or positively depending on whether the animals were stress resilient or whether they had developed a maladaptive response to stress (Lagace et al., 2010). To complicate matters further, it has also been shown that chronic foot shock stress has differential effects on hippocampal neurogenesis in adult rats as a function of their sex and housing (single versus grouped) (Westenbroek et al., 2004). Thus, when interpreting data on stress-induced neurogenesis, it is crucial to take into consideration the type of stressor, the duration of stress, the species and sex of the animals as well as the timing post stressor at which the different stages of neurogenesis were evaluated. The precise mechanisms underlying the stress-mediated effects on neurogenesis remain to be unveiled. Studies now suggest that both adrenal hormone pathways in combination with the regulation of the neurotransmitter systems are involved (Veena et al., 2011).

Table 1.3: Stress and adult neurogenesis. Adapted from (Veena et al., 2011).

Type of stressor	Species	Acute vs. chronic (duration)	Effect on neurogenesis	Reference
Predator odour	Male SD rats	Acute (1 hour)	Proliferation ↓ Survival & Differentiation ↔	(Falconer and Galea, 2003; Tanapat et al., 2001)
	Female SD rats	Acute (1 hour)	Proliferation ↔ Differentiation↔	(Falconer and Galea, 2003)
Psychosocial stress	Adult tree shrews	Acute (1 hour)	Proliferation ↓	(Gould et al., 1997)
	Male SD rats	Acute (1 day)	Proliferation ↔ Survival & Differentiation ↓	(Thomas et al., 2007)
	Male tree shrews	Chronic (7+28 days)	Proliferation ↓	(Czeh et al., 2001)
Residential intruder stress	Marmoset monkeys	Acute (1 hour)	Proliferation ↓	(Gould et al., 1998)
Restraint stress	SD rats	Chronic (6 hours/day for 3 weeks)	Proliferation ↓ Survival ↓ (slight) Differentiation ↔	(Pham et al., 2003)
	SD rats	Acute (2 hours and 6 hours, single session)	Proliferation ↔	
	SD rats	Acute (4 hours/day for 7 days)	Proliferation ↓	(Luo et al., 2005)
	Male SD rats	Acute (3 hours, single session)	Proliferation ↓	(Bain et al., 2004)
	C57BL/6J mice	Acute (3 hours, single session)		
	Male SD rats	Chronic (6 hours/day for 14 days)	Proliferation ↓	(Xu et al., 2006)
	Male Wistar rats	Chronic (6 hours/day for 21 days)	Proliferation ↓ Survival ↓ Differentiation ↓	(Veena et al., 2009a; Veena et al., 2009b)
Cold immobilization & forced swimming	Male Wistar rats	Acute (1 hour and 30 min respectively)	Proliferation ↔	(Heine et al., 2004a)

Type of stressor	Species	Acute vs. chronic (duration)	Effect on neurogenesis	Reference
Early life stress	SD rats	Chronic (maternal separation for 1 week)	Proliferation ↓	(Lee et al., 2001; Park et al., 2002)
	SD rats	Chronic (maternal separation for 15 min from P1 to P14)	Proliferation ↔ Survival ↔ Differentiation ↔	(Mirescu et al., 2004)
	SD rats	Chronic (maternal separation for 180 min from P1 to P14)	Proliferation ↓ Survival ↔ Differentiation ↔	
Prenatal stress	SD rats	Chronic (restrained stress of dam for 45 mins daily from E15 to delivery day)	Proliferation ↓ Differentiation ↔	(Lemaire et al., 2000)
	Adult rats	Chronic (unpredictable daily stress of dam during last week of pregnancy)	Proliferation ↓ Differentiation ↓	(Mandyam et al., 2008)
Unpredictable stress	Male Wistar rats	Chronic (daily for 3 weeks)	Proliferation ↓ Survival ↔ Differentiation ↔	(Heine et al., 2005)
	Male Wistar rats	Chronic (daily for 3 weeks)	Proliferation ↓ Survival ↓	(Oomen et al., 2007)
Mild stress	Swiss albino mice	Chronic (daily for 5 weeks)	Proliferation ↓	(Li et al., 2008a)
	SD rats	Chronic (daily for 19 days)	Proliferation ↔ Survival ↓ Differentiation ↔	(Lee et al., 2006)
	Male Wistar rats	Chronic (daily for 2 weeks)	Proliferation ↓ Survival ↓ Differentiation ↓	(Silva et al., 2008)
Intermittent stress	Male SD rats	Chronic (6 hours of restraint per day for 14 days)	Proliferation ↓	(Rosenbrock et al., 2005)

Type of stressor	Species	Acute vs. chronic (duration)	Effect on neurogenesis	Reference
Isolation stress	Mice	Chronic (from time of weaning)	Proliferation ↓	(Dong et al., 2004)

1.8 Neuroinflammation

Neuroinflammation is defined as the complex biological response of the nervous tissue to harmful stimuli such as pathogens, damaged cells, toxic metabolites, infection, traumatic brain injury or autoimmunity. As such it protects the CNS by employing immune cells, molecular mediators such as cytokines, and blood vessels to eliminate the cause of insult, and commence tissue repair (Gendelman, 2002). In the last decades neuroinflammation has gained a lot of attention as it has been positioned as a main protagonist in neuronal degeneration and hippocampal dysfunction under normal ageing or pathological conditions. Neuroinflammation is evident in many pathological conditions, including multiple sclerosis, ischemic stroke and neurodegenerative disorders such as Alzheimer's disease, PD and age-related dementia. It has been associated with the cognitive decline observed in patients suffering from these conditions (Amor et al., 2014; Green and Nolan, 2014; Leonard, 2007; Ryan and Nolan, 2016). Hippocampal neurogenesis has been shown to be negatively affected by neuroinflammation (Ekdahl et al., 2009; Heine et al., 2004a; Monje et al., 2003). Moreover, neurogenesis has been associated with impaired hippocampal-dependent functioning as well as implicated in the pathologies underlying neurodegenerative and psychiatric conditions (Amor et al., 2014; Barrientos et al., 2015; Kohman and Rhodes, 2013; Ryan and Nolan, 2016). It is believed that understanding the interplay between hippocampal neurogenesis, neuroinflammation and cognitive function will provide a valuable insight into tackling challenges faced during normal ageing as well as therapeutic strategies for undertaking neurodegenerative and psychiatric disorders (Fan and Pang, 2017; Villeda et al., 2011).

1.8.1 Microglia

Microglia are the resident immune cells of the CNS (Gendelman, 2002). They actively survey the brain parenchyma and respond rapidly upon detecting signs of injury (Garden, 2013). In the case of acute inflammation in the CNS, microglia are the sole mediators of the immune response (Streit et al., 2004). However, if the inflammation persists and becomes chronic, it can cause the deterioration of tissue and most importantly of the BBB, a highly selective semipermeable border consisting of astrocytes, endothelial cells and pericytes, that separates the CNS from circulating blood and peripheral immune cells (Daneman and Prat, 2015). Thus, during chronic inflammation microglia release signals towards the peripheral immune cells which then become involved in dealing with the inflammatory challenge (Garden, 2013; Streit et al., 2004).

1.8.1.1 Origin and development of microglia

Microglia originate from immature uncommitted erythromyeloid progenitors found in the yolk sac of mice embryos. The erythromyeloid progenitors are a type of stem cell that are formed during primitive haematopoiesis, or the transient early wave of myeloid cell development, which occurs at E7.5 to E8 (Kierdorf et al., 2013). These cells start to upregulate CD45, which is a common antigen present on all haematopoietic cells and a key player in cell activation (Huntington and Tarlinton, 2004). They also upregulate F4/80, which is a macrophage receptor protein member of the adhesion G protein-coupled receptor family (Lin et al., 2005) and is known as an A1 type of cell ((CD45+/F4/80+); See Figure 1.9). Subsequently, A1 cells differentiate into A2 cells, which express more myeloid cell markers such as CX3C

chemokine receptor 1 (CX3CR1) and colony-stimulating factor 1 receptor (CSF1R), and begin to populate the brain mesenchyme (Kierdorf et al., 2013). The A2 cells have been observed on the surface of the developing mouse brain at E9 (See Figure 1.9 (Prinz and Priller, 2014)). These cells present an amoeboid shape and at E13.5 a large population of them can be detected in the lining of the fourth ventricle of the mouse embryo (Chan et al., 2007). These resident microglial cells are said to expand 20-fold in the first postnatal days in the rodent (P0-P11), and evidence suggest that they remain in the brain throughout life and sustain the microglial population locally (Alliot et al., 1999; Ginhoux et al., 2010; Prinz and Priller, 2014; Schulz et al., 2012).

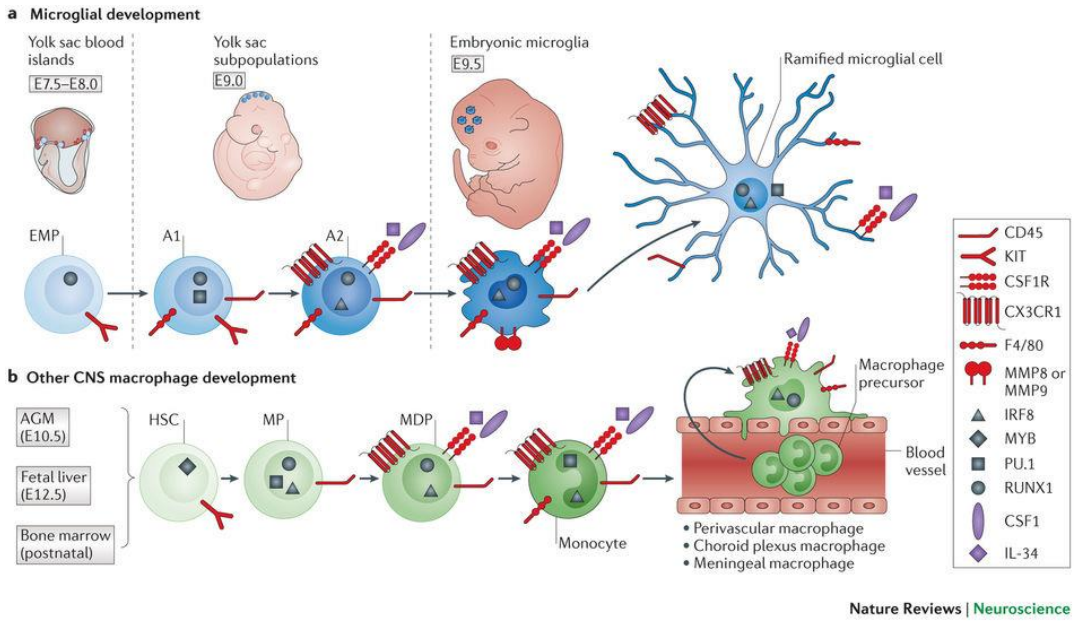

Figure 1.9: Origin and development of microglia and other CNS microphage cells. Adapted from (Prinz and Priller, 2014).

Human microglia appear to share the pattern of development observed in rodent microglia, in that microglial-like cells with different morphologies have been identified in human foetuses as early as 3 weeks post gestation (Hutchins et al., 1990). Nonetheless, the maturation period for human microglia appears to span across the full gestational period with the first microglia observed in the spinal cord around week 9.

The major microglia influx and colonization of the developing CNS occurs around week 16, and a wide spread of ramified microglia is evident in the intermediate zone by week 22 (Prinz and Priller, 2014; Rezaie et al., 2005; Rezaie and Male, 1999). A well-defined population of differentiated microglia has been observed to fully spread across the developing human brain between week 35 and term (Esiri et al., 1991; Prinz and Priller, 2014; Verney et al., 2010).

Based on their origin and development, microglia represent a completely distinct population of cells from other macrophages observed in the CNS such as the perivascular, choroid plexus and meningeal macrophages. These other macrophages originate from the process of definitive haematopoiesis, which begins at E10.5 in the rodent aorta-gonad-mesonephros, and at E12.5 in the foetal liver, and continues postnatally in the bone marrow, where monocytes are formed. During definitive haematopoiesis, hematopoietic stem cells give rise to myeloid precursors, from which monocytes are derived, and to macrophage and/or dendritic cell progenitors (See Figure 1.8; reviewed by (Prinz and Priller, 2014)).

1.8.1.2 Function of microglia

Microglia have a wide range of functions across the lifespan and across different regions of the CNS (reviewed by (Boche et al., 2013)). To begin with, during CNS development microglia contribute to the formation of neuronal circuits and promote their survival through releasing neurotrophins, growth factors and cytokines (Deverman and Patterson, 2009; Nayak et al., 2014). Furthermore, during CNS

development, microglia have been shown to prune redundant neurons by initiating cell death programmes followed by phagocytosis, or to clear cellular debris after apoptosis. They have also been shown to promote NPC survival in the developing CNS, to engulf less active intact synapses and to regulate activity-dependent synaptic remodelling (Boche et al., 2013; Schafer and Stevens, 2015). Furthermore, microglia are involved in remodelling of the extracellular matrix to facilitate cell migration and cell interactions which promotes tissue turnover during normal development and regulates physiological signalling during inflammation (Milner and Campbell, 2003; Stamenkovic, 2003; Vincent et al., 2012). Microglia are functional modulators of the inflammatory and immune responses of other cells in the CNS through the secretion of chemoattractant molecules to signal to other immune cells (Salvi et al., 2017). They can detect immune signals from neuronal cells through different pathways including the CD200/CD200R pathway (Oria et al., 2018; Walker and Lue, 2013), and by promoting further activation of immune cells through interferon-γ (IFNγ) secretion (Kreutzberg, 1996; Sochocka et al., 2017). In addition to regulating stem and progenitor cells in the developing CNS, microglia are also involved in the regulation of adult NPCs in the hippocampus. Further, they secrete lipoprotein particles which provide lipids to neurons and aid in maintenance of cell membranes and synapses as well as to facilitate synaptic plasticity (Boche et al., 2013; Wang and Eckel, 2014). Finally, microglia also mediate tissue repair to facilitate plasticity and synaptogenesis (Jin and Yamashita, 2016; Michell-Robinson et al., 2015).

The innate immune function of microglia is to recognise pathogens, which they accomplish through Toll-like receptor-mediated (TLRs) release of cytokines and chemokines in response to pathogens and endotoxins such as the lipopolysaccharide (LPS: a frequently used pathogen in *in vitro* and *in vivo* models of acute inflammation) (Boche et al., 2013; Facci et al., 2014; Yao et al., 2013). The Toll-like receptors present on microglia recognize evolutionary conserved antigens on the surface of pathogens (pathogen-associated molecular patterns) and have been shown to induce microglial activation and release of pro-inflammatory cytokines under various pathological conditions such as in response to the herpes simplex virus (Aravalli et al., 2005; Marinelli et al., 2015; Mogensen, 2009). Similar mechanisms of microglial activation are assumed to occur in response to accumulation of extracellular protein such as β-amyloid (Doens and Fernandez, 2014; Tahara et al., 2006). Thus, another primary function of microglia is to ingest and destroy groups of damaged cells due to infarct (Weinstein et al., 2010); neuronal cells due to neuronophagia (Troost et al., 1993), Wallerian degeneration (Rotshenker, 2011) and tract degeneration (Koshinaga and Whittemore, 1995) through their digestive enzymes and lysosomes (phagocytosis). Microglia have been shown to further phagocytose micro-organisms in the brain such as in abscess (Kielian, 2004; Mariani and Kielian, 2009), virally infected cells in the case of herpes encephalitis (Lafaille et al., 2015; Lokensgard et al., 2002; Schachtele et al., 2010) and erythrocytes and haemoglobin breakdown products such as hemosiderin following haemorrhage (Taylor and Sansing, 2013; Yang et al., 2016). Microglia play an important role in the process of antigen presentation, where pathogens from viral, bacterial or fungal origin bind to the microglial receptors major histocompatibility complex (MHC) class I and II and thereby proceed to the

recruitment of T cells and lymphocytes (involved in the adaptive immune response) to eliminate the cause of inflammation (Almolda et al., 2011; Mogensen, 2009; Shaked et al., 2004), a process that may be implicated in the development of autoimmune diseases (Boche et al., 2013; Mogensen, 2009).

Microglia can also exhibit some harmful functions within the CNS (Boche et al., 2013). For instance, they have been shown to contribute to the myelin destruction in multiple sclerosis (Luo et al., 2017). They have also been shown to support mycobacterium tuberculosis and facilitate its survival in the CNS (Rock et al., 2005; Spanos et al., 2015). Furthermore, microglia express receptors that allow the entry and replication of HIV-1 viruses into the CNS (Joseph et al., 2015). Interestingly, even though microglia (the resident immune cell) are designed to prevent the CNS from malignancies, it has been suggested that with tumour and metastases progression microglia can be polarized into becoming tumour-supportive and immunosuppressive cells, thereby contributing to tumour growth (Graeber et al., 2002; Wu and Watabe, 2017). Additionally, microglia can induce cytotoxicity and impair otherwise healthy cells when they produce abnormal levels of reactive oxygen species (ROS), pro-inflammatory cytokines, or abnormal secretion of glutamate and aspartate (Banati et al., 1993; Boche et al., 2013).

1.8.2 Microglia and hippocampal neurogenesis

Microglia shape adult hippocampal neurogenesis in a multitude of ways. Specifically, they phagocytose newborn neurons that fail to integrate into existing circuitry (Sierra

et al., 2010), they regulate glutamatergic receptors maturation and synaptic transmission, and support synaptic pruning (Sierra et al., 2014a). Additionally, microglia can suppress neurogenesis under inflammatory conditions by exhibiting a neurotoxic phenotype (Belarbi and Rosi, 2013). Such opposing roles are explained to some extent by the distinct polarization states of activation that microglia can adopt.

1.8.2.1 Polarization/Activation patterns in the context of hippocampal neurogenesis

Quiescent (also known as ramified or resting) microglia have a distinct morphology – a small cell body and multiple extending processes, with which they probe the environment for signals of injury, trauma or other disruption of homeostasis. *In vitro* studies have shown that in this resting state, microglia release factors which rescue neuroblasts and instruct neuronal cell differentiation. Furthermore, through the release of unidentified factors, microglia enhance and prolong the neurogenic potential of cultured cells (Kohman and Rhodes, 2013). When presented with an inflammatory challenge, microglia exhibit a classically activated (also known as M1 or neurotoxic) pro-inflammatory phenotype. The M1 phenotype is defined by an amoeboid and slightly enlarged cell body and a reduced number of short processes. The markers expressed by pro-inflammatory microglia include CD68, iNOS, TNFα, IL-1β, and COX2 (Franco and Fernandez-Suarez, 2015). In this state microglia have negative effects on hippocampal neurogenesis through decreasing the survival of newborn neurons (Ekdahl et al., 2003), impeding proliferation (Fujioka and Akema, 2010), decreasing neuronal differentiation and impairing successful integration of newborn neurons in the existing hippocampal circuitry (Belarbi et al., 2012). The alternatively

activated microglia (also known as M2 or neuroprotective), on the other hand, are involved in regeneration. The adoption of an alternatively activated phenotype has been shown to follow the expression of a classical activated phenotype, potentially serving as a compensatory response to aid repair. For instance, it has been shown that while M1 microglia reduce neuronal differentiation and survival *in vitro*, M2 enhance both of those processes (Kohman and Rhodes, 2013). In an animal model of Alzheimer's disease, when stimulated towards adopting an M2 phenotype, microglia alleviated the suppression of neurogenesis that was caused by neuroinflammation (Kiyota et al., 2012). Though similar in morphology, the M2 phenotype can be separated from the M1 phenotype by a distinctive pattern of marker expression. In their alternatively activated state, microglia can be identified by the following markers: IL-10, TGF-β, Arg1, Mannose receptor type 1. Across all three states, microglia are immunoreactive to Iba-1 (calcium-binding protein), and this is thus a useful method for studying their morphology (Franco and Fernandez-Suarez, 2015). Worth noting is the fact that scientists have more recently suggested that microglial polarization is a much more complex process and rather works in a gradient fashion spanning between the defined M1 and M2 polarizations and that the M2 phenotype can adopt variations (termed M2a. M2b, M2c, M2d). Thus, microglial activation works on a spectrum between a highly pro-inflammatory phenotype to an anti-inflammatory M2 phenotype (Colton and Wilcock, 2010; Zhou et al., 2017). Taking this into account will further our understanding of how microglia activation affects processes in the brain such as hippocampal neurogenesis under physiological and pathological conditions.

1.8.2.2 The different effects of microglia on adult hippocampal neurogenesis

Microglia are suggested to perform the following three roles in the neurogenic niche: they prime NPCs towards neuronal differentiation; they enhance NPC proliferation; they promote the survival of newborn neurons (reviewed by (Gemma and Bachstetter, 2013). The supportive roles of microglia to adult SGZ neurogenesis are performed mostly when the cells are in their ramified non-activated state. Sierra and colleagues established that a crucial role of microglia in the neurogenic niche is to phagocytose the apoptotic cells during the phase of early survival through an immunologically silent process, i.e. one without inflammation, where microglia do not adopt amoeboid or activated state (Sierra et al., 2010). This process of microglial phagocytosis is distinct from that which occurs during tissue repair or tissue damage, after traumatic brain injury for instance (Gemma and Bachstetter, 2013). Furthermore, Sierra and colleagues demonstrated that the phagocytosis that takes place within the neurogenic niche is performed by the microglial processes, rather than the cell body (during inflammation) such that the end processes of microglia form phagocytic pouches that engulf the apoptotic cells. These pouches ensue at terminal branches autonomous from the microglial soma (Sierra et al., 2010). This is important property, given the huge number of neuroblasts generated daily and the small percentage of them surviving and integrating into the circuitry. Microglia have thus developed a very efficient way of clearing the large proportion of apoptotic cells by employing a comparatively smaller proportion of their own population and achieving the task in timely manner in accordance with the rate of the neurogenic process (Gemma and Bachstetter, 2013; Sierra et al., 2010).

Soluble factors secreted from microglia can directly affect embryonic and adult mouse NPCs. Microglial-conditioned media collected from rat primary microglial cultures displayed a very strong positive effect on the rat primary neuronal cultures that were treated with it for 7 days *in vitro*. Not only the neuronal proliferation measured by Ki67 immunopositivity was enhanced, but also the viability and survival of the neurons was increased by 50% when microglia-conditioned media was used compared to normal media (Morgan et al., 2004).These results were replicated in a SVZ-derived NPC culture (Walton et al., 2006). It has been proposed that ramified microglia can mediate neurogenesis through the production of IGF-1 and BDNF (Ziv and Schwartz, 2008) and a summary of the ways ramified microglia support hippocampal neurogenesis is presented in Figure 1.10 (Gemma and Bachstetter, 2013).

It has been demonstrated that inhibition of adult rat SGZ neurogenesis by LPS-induced inflammation is mediated through microglial release of the cytokines IL-6 and TNFα (Monje et al., 2003). Furthermore, direct evidence for the detrimental role of microglia in inflammation-induced suppression of neurogenesis, came from a seminal study where minocycline, which blocks microglial activation, prevented the negative effect of LPS on hippocampal neurogenesis (Ekdahl et al., 2003). Additionally, the decrease in neurogenesis observed during ageing (Seib and Martin-Villalba, 2015) coincides with changes in the phenotype of hippocampal microglia, which appear to adopt a more inflammatory state (Dilger and Johnson, 2008). With age, microglia are primed towards a prolonged pro-inflammatory response, and thus exaggerated sickness

behaviour and cognitive deficits occur in response to an immune challenge (Dilger and Johnson, 2008; Kohman, 2012). Direct evidence for the contribution of microglia to the reduced neurogenesis observed during ageing comes from a study illustrating that microglia-secreted IL-1β led to reduced hippocampal neurogenesis in aged rats and that blocking the cleavage of endogenous IL-1β prevented the age-related decrease in neurogenesis (Gemma et al., 2007). Additionally, aged mice presented with an increase in cell proliferation as well as an increase in pro-inflammatory phenotype of hippocampal microglia. Interestingly, both of these phenomena were attenuated after the aged mice were exposed to a voluntary exercise paradigm (Kohman et al., 2012). However, it is important to note also that when an *in vitro* preparation of hippocampal NPC cultures of sedentary mice was treated with microglia from exercising mice, the number of proliferating precursors increased dramatically, suggesting that microglia were primed towards a proneurogenic phenotype by the enriched experience (Vukovic et al., 2012). Nonetheless, this finding has been contested by a study employing a different methodology (see (Gebara et al., 2013)), thus the precise role of microglia under conditions of enriched environment remains to be elucidated.

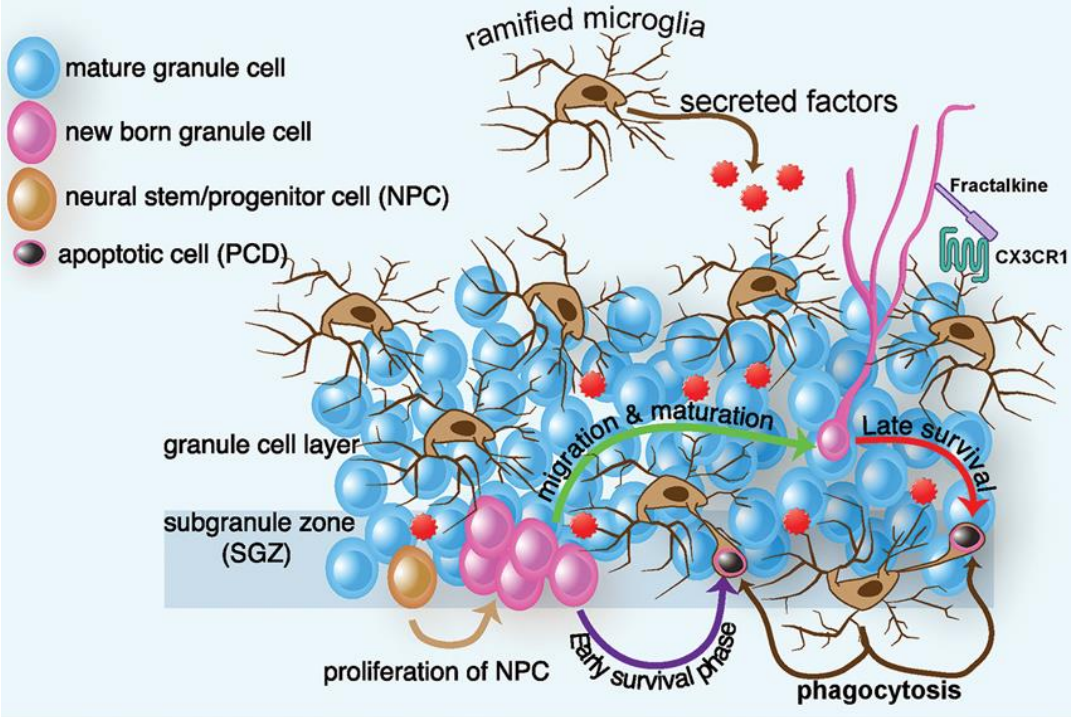

Figure 1.10: Ramified microglia modulate hippocampal neurogenesis. Adapted from (Gemma and Bachstetter, 2013).

1.8.2.3 Microglial-neuronal cell interaction

At least two pathways through which microglia-neuronal communication occurs have been identified. A membrane protein exclusively expressed by microglia is the CD200 receptor (CD200R). Its ligand CD200 is expressed by neurons, astrocytes and oligodendrocytes. In a transgenic mouse model, in which CD200 expression was silenced, LTP was impaired (Bechade et al., 2013). Another membrane protein, the CX3C receptor-1 (CX3CR1) is expressed by microglia and some macrophages, and its ligand is the neuronally-derived chemokine CX3CL1 (fractalkine). In transgenic mice with a functional deletion of CX3CR1, hippocampal- and olfactory-dependent memory was improved coupled with increased number and soma size of hippocampal, but not olfactory microglia (Reshef et al., 2014). This finding was replicated after pharmacological inhibition of CX3CL/R1 signalling. The microglia exhibited a mildly

activated status as defined by increased cell density and soma size. The authors thus propose an inverted U model explaining the interaction between hippocampal functioning and microglia activation – with high and low activation being detrimental to hippocampal-dependent tasks, while moderate increase in microglia cell density led to improved performance (Reshef et al., 2014). Additionally, adult rats exhibited decreased hippocampal neurogenesis when either CX3CR1 was genetically deleted or pharmacologically inhibited (Bachstetter et al., 2011). Moreover, the authors noticed that in their aged cohort of animals, the decrease in hippocampal neurogenesis was coupled with a decrease in fractalkine levels, and administration of the latter resulted in a reinstated ramified phenotype of the aged microglia as well as rescued levels of neurogenesis in the aged hippocampus (Bachstetter et al., 2011). Interestingly, in the absence of intact CX3CL1/CX3CR1 signalling, impairments in motor learning, cognitive functioning and synaptic plasticity, specifically LTP, have been observed (Rogers et al., 2011). Nonetheless, using the same mouse model, other researchers did not find any impairments in LTP. On the contrary, upon administration of fractalkine they found LTP to be inhibited (Maggi et al., 2009). Thus, local and specific interactions between neurons and microglia can play an important role in the modulation of neuronal function and adult neurogenesis. Thus, understanding the intricate relationship by which microglia and neurons can tune each other's activity is an important priority for this field.

1.8.3 Cytokines and hippocampal neurogenesis

Cytokines are a category of small proteins, released by immune cells, that play important roles in inter- and intra- cellular as well as endocrine signalling in the

organism (Kim et al., 2016b). In the CNS, cytokines are mainly released by microglia and astrocytes, although peripherally-released cytokines have been shown to cross the BBB and infiltrate the CNS under inflammatory conditions (Lucas et al., 2006; Tansey and Wyss-Coray, 2008). The main role of cytokines is in communicating signals between neuronal and immune cells in the CNS and depending on the cytokine released, the signals can be generally classified as either pro- or anti- inflammatory (Kim et al., 2016b). It is important to bear in mind, however, that such a polarized division of cytokines does not accurately represent their function as harmful or beneficial. It should be taken into account the type of cell releasing the cytokine, the concentration in which the cytokine is found, the type of cell that the cytokine is targeting as well as the timing and type of the activation signal (Cavaillon, 2001). These factors also play role in the influence that cytokines have on hippocampal neurogenesis (See Table 1.4; reviewed by (Kohman and Rhodes, 2013)).

The proinflammatory mediator interleukin-6 (IL-6) is primarily secreted in the brain under pathological conditions. In a transgenic mouse model where astrocytes chronically produced IL-6, it was found that neurogenesis in the adult hippocampus was dramatically reduced (by 63%) (Vallieres et al., 2002). Moreover, all stages of the neurogenic process were influenced, namely cell proliferation, differentiation and survival, without affecting cell apoptosis or changing the morphology of either astrocytes or microglia, thus indicating that the IL-6 concentration itself was not toxic to the brain environment in general (Vallieres et al., 2002). Another study found that

upon LPS-induced microglial activation, microglia inhibited hippocampal neurogenesis in an IL-6- mediated manner (Monje et al., 2003).

Tumour necrosis factor α (TNFα) has been reported to have similar negative effects as IL-6 has on hippocampal neurogenesis. For example, chronic exposure to TNFα was found to decrease the proliferation of NPCs within the SGZ (Seguin et al., 2009). Additionally, *in vitro* treatment with TNFα under differentiation conditions resulted in increased apoptosis of hippocampal NPCs (Hofer et al., 2011). Interestingly, in another *in vitro* model of hippocampal NPC grown under differentiation conditions, administration of TNFα was found to impair neuronal differentiation, but not proliferation, of the NPCs (Keohane et al., 2010). What is more, the authors found that the decrease in neuronal differentiation was on account of increased astrocyte differentiation, due to an increase in expression of an anti-neurogenic gene Hes1 (Keohane et al., 2010). Nonetheless, the two studies used different doses of TNFα, which may explain the differences in NPC proliferation observed by the researchers. The findings on the displaced proportion of neuronal-to-astrocyte differentiating NPCs of Keohane and colleagues were in agreement with the previous work of Johansson et al. who showed that treating human and mouse NSPC cell lines with TNFα resulted in increased astrocytic differentiation and decreased neuronal differentiation of the precursors (Johansson et al., 2008). Lastly, a transgenic mouse line lacking the type 1 receptor for TNFα exhibited an increased baseline rate of proliferating NPCs in the SGZ suggesting that TNFα mediates its effects through binding its cognate receptor (Iosif et al., 2006).

As well as detrimental cytokine-driven effects on neurogenesis, some cytokines have been shown to stimulate hippocampal neurogenesis. For instance, upon *in vitro* stimulation of microglial cells with the anti-inflammatory interleukin-10 (IL-10), NPCs in the culture exhibited increased cell proliferation while no effect on differentiation was observed (Kiyota et al., 2012). In another *in vitro* model where microglia were actively secreting IL-10, it was found that neuronal differentiation and survival was positively enhanced (Cacci et al., 2008). Lastly, the positive effects of IL-10 were also apparent in a mouse model of Alzheimer's disease, where overexpression of IL-10 rescued the neurogenic deficits observed such that cell proliferation, neuronal differentiation and cell survival were all positively affected from the treatment, without affecting the accumulation of plaques (Kiyota et al., 2012).

IL-1β has perhaps gained the most attention in relation to its involvement in hippocampal neurogenesis (reviewed by (O'Leime et al., 2017)). Increased levels of circulating IL-1β have been suggested to alter neurogenesis by decreasing cell proliferation and priming differentiation towards a non-neuronal fate (Kohman and Rhodes, 2013). IL-1β is secreted by microglia and astrocytes and its type 1 receptor (IL-1R1) has been shown to be expressed on NPCs (Green and Nolan, 2012a; Green and Nolan, 2012b; Green et al., 2012). In an *in vitro* model of rat embryonic hippocampal neurospheres, IL-1β treatment led to a significant reduction in the number of proliferating NPCs and the growth rate of the neurospheres (Green et al., 2012). What is more, when the researchers cultured the neurospheres under

differentiation conditions in the presence of IL-1β, the cells differentiated largely into astrocytes, and few neurons arising from the NPCs were observed (Green et al., 2012). In an *in vivo* study employing a conditional transgenic mouse model in order to overexpress IL-1β selectively in the hippocampus of the mice, the authors observed a significant reduction in the number of DCX+ cells in the GCL following 1 and 3 months of overexpression (Wu et al., 2012). The outcome of differentiating cells in the SGZ in this study replicated the *in vitro* findings of Green and colleagues, with the mice overexpressing IL-1β displaying a significantly higher proportion of newborn astrocytes rather than neurons. The authors further showed that the IL-1β overexpression effects were mediated through an interaction with the IL-1R1 expressed on the hippocampal NPCs, since in transgenic mice where IL-1R1 was absent, overexpression of IL-1β had no effect on the number of DCX+ cells or the population of differentiating NPCs (Wu et al., 2012). IL-1β has also been positioned as a potential mediator of the stress-induced decreases in neurogenesis (Koo and Duman, 2008), the LTP impairments observed in CX3CR1 deficient mice (Rogers et al., 2011), and has been shown to regulate the expression of the master regulator of neurogenesis TLX in a time- and dose-dependent manner (Green and Nolan, 2012b).

Table 1.4: Cytokines and hippocampal neurogenesis. Adapted from (Kohman and Rhodes, 2013).

Cytokine	Proliferation	Survival	Neuronal differentiation	Astrocyte Differentiation	Reference
IL-6	↓	↓	↓		(Monje et al., 2003; Vallieres et al., 2002)
IL-1β	↓		↓	↑	Green et al. (2012) Koo and Duman (2008)
TNFα	↓		↓	↑	(Iosif et al., 2006; Johansson et al., 2008; Keohane et al., 2010; Seguin et al., 2009)
IL-10	↑				(Cacci et al., 2008; Kiyota et al., 2012)

1.9 Adolescence

Adolescence is a critical phase of development associated with hormonal and behavioural changes that are preserved across species (Crews et al., 2016). The precise age boundaries of adolescence in rodents have been debated (Spear, 2000b) but most recently, it has been agreed that early adolescence or the juvenile period takes place between approximately P21 to P42, while late adolescence and transitioning towards early adulthood takes place between P43 and P60 (Laviola et al., 2003; Spear, 2015). The early and late adolescent periods in rodents correspond to 10-18 and 18-25 years of age in humans, respectively (Crews et al., 2016). Sexual maturation occurs during the adolescent period for both humans and rodents and despite differences in the specific hormonal and physiological changes that occur, females of both species mature earlier than males (P36 and P44 for female and male rats correspondingly;

(Vetter-O'Hagen and Spear, 2012). Interestingly, certain behavioural patterns characteristic of the adolescent period are also conserved across species, namely elevated reward seeking and risk taking behaviour, increased affiliative behaviour and social interactions, such as play and grooming for rodents (Douglas et al., 2004), elevated novelty seeking behaviour (Douglas et al., 2003), as well as diminished responsivity to aversive stimuli, such as fear conditioning for rodents (Spear, 2000a; Spear, 2011). Notably, adolescence is a key period for susceptibility to stress and inflammation and an extremely sensitive period for the onset of many neurological and psychiatric conditions such as epilepsy, neurodegeneration and neuro-atrophy (Macleod and Appleton, 2007), as well as anxiety-, addiction-, mood- related disorders and schizophrenia (Green and Nolan, 2014; Kessler et al., 2005; O'Connor and Cryan, 2014; Paus et al., 2008). Thus, adolescence is a critical period of development and deeper understanding of the processes taking place during this time will contribute to the knowledge of mechanisms underlying various neuro-psychiatric disorders.

1.9.1 The adolescent brain

Throughout adolescence, important changes in the brain such as plasticity-driven organization of neural circuits within and in between limbic and cortical areas occur (Pattwell et al., 2011; Selemon, 2013). For instance, during early adolescence or the juvenile period, axons and synapses in various areas have been overproduced (Crews et al., 2007). Further structural changes in later adolescence including decreases in grey matter coupled with increases in white matter following anterior-to-posterior trajectory (Mengler et al., 2014; Oguz et al., 2013) have been associated with synaptic pruning, increased myelination of existing axons, as well as development of the

extracellular matrix (Paus et al., 2008; Rakic et al., 1994). Subcortical limbic structures such as the hippocampus and the amygdala as well as the nucleus accumbens mature before the cortical areas, which can account for the impulsive risk-taking and reward-seeking behavioural characteristics of adolescence (Agoglia et al., 2017; Casey et al., 2008; Crews et al., 2016; Geier et al., 2010). Within the cortex, sensorimotor cortices mature first while the PFC continues its development into early adulthood (Cunningham et al., 2002). During adolescence, the visual and sensorimotor cortices undergo critical changes during which they are extremely plastic and responsive to enrichment or stimulation, which ultimately determines their synaptic rearrangements and maturation (Gordon and Stryker, 1996). It is important to note that such experience-driven synaptic formation and plasticity as well as synaptic pruning and extracellular matrix degradation at later stages of development involve neuronal-glial (specifically complement-dependent microglial) signalling (Schafer et al., 2012; Tremblay et al., 2010). Concurrent with cellular changes, neurotransmitter levels also change within the cortical and subcortical areas during adolescence. A spike in dopamine and dopaminergic receptor expression occurs in the hippocampus and nucleus accumbens during early adolescence (Tarazi and Baldessarini, 2000), followed by the development of dopamine projections to the PFC and spike of dopamine within the PFC at later adolescence (Kalsbeek et al., 1988). Dopaminergic levels normalize in the subcortical and cortical areas during later adolescence and early adulthood, respectively (Crews et al., 2016). Similar patterns of overexpression followed by decline is observed in other neurotransmitter systems innervating the hippocampus. For instance with respect to the inhibitory GABA, maturation of the $GABA_A$ receptor is stagnant during adolescence but progresses during adulthood (Yu

et al., 2006), while the maturation of $GABA_B$ is completed during mid-adolescence (Nurse and Lacaille, 1999). Receptor expression of the excitatory glutamatergic neurotransmitter are markedly decreased during adolescence (Insel et al., 1990), while the opposite pattern is observed in the expression of the serotonergic 5-HT terminals (Xu et al., 2001). In rodents, by approximately P30, myelogenesis, DG formation and cerebellar neurogenesis are completed, thus restricting neurogenesis in the adolescent brain to the niches where the process persists through adulthood – the SVZ and the SGZ of the DG (Lemasson et al., 2005; Li et al., 2009).

1.9.2 Influences of stress and exercise on hippocampal neurogenesis during adolescence

Adolescence involves many physiological and behavioural transitions that can incur significant stress (Spear, 2000a). In rodents, relatively few studies have examined the impact of stress on hippocampal neurogenesis (reviewed by (Hueston et al., 2017)). Exposing male and female adolescent rats to chronic stress for a period of 15 days (social instability stress P30-P45) resulted in differential regulation of hippocampal neurogenesis, immediately after cessation of the stress, across the sexes. Specifically, there was an increased number in proliferating cells and newborn neurons in males (McCormick et al., 2012) but reduced number of neuronal survival and no change in number of proliferating cells in females (McCormick et al., 2010). Interestingly, five-weeks of social isolation in male mice, starting at P24, induced a decrease in newborn neuronal survival and neuronal differentiation of NPCs within the DG, without inducing alterations in cell proliferation (Ibi et al., 2008). Additionally, social isolation for either a 1 or 3 week period during adolescence was associated with a decrease in

cell proliferation and neurogenesis within the DG of non-human primates (Cinini et al., 2014). DG volume and neuronal density in the rat DG were not affected when measured in late adolescence (P56) after a 4-week exposure to chronic physical and social stress (Isgor et al., 2004). However, LTP was decreased within the CA1 of juvenile rats immediately after they had undergone elevated platform stress (Xiong et al., 2004). Taken together and despite the fact that the last two studies used indirect measures of neurogenesis (DG volume (Isgor et al., 2004) and LTP (Xiong et al., 2004)), it appears that hippocampal neurogenesis during adolescence can be either enhanced or inhibited by stress and this is contingent on the sex of the animals, the timing (early versus late adolescence) and the type of stressor. With respect to the lasting effects of the experience of stress during adolescence on adult hippocampal neurogenesis, findings converge on stress being associated with impaired neurogenesis in males examined during late adulthood (Sterlemann et al., 2010) and females during late adolescence/early adulthood (Barha et al., 2011), but increased neurogenesis and cell survival when examined during early adulthood in males (Barha et al., 2011; Hueston et al., 2017; McCormick et al., 2012).

The impact of adolescent stress on hippocampal-dependent and neurogenesis-associated behaviours in rodents remains relatively unexplored. Male rats who underwent social isolation stress from early to late adolescence, performed poorly on a memory test (NOR) when tested upon stress cessation (Bianchi et al., 2006). Socially isolated male mice, throughout the adolescent period, also exhibited a similar memory impairment tested using the MWM at the verge of late adolescence/early adulthood

(P59; (Ibi et al., 2008)). Nonetheless, when adolescent rats were exposed to other stressors, such as chronic variable physical or social stress, they did not exhibit any memory impairment in late adolescence when tested on the MWM task (Isgor et al., 2004). Thus, the type of stressor is critical to whether adverse effects on cognitive performance would be observed during late adolescence. Upon examination of the cognitive performance in adulthood after adolescent rats have been exposed to different types of acute stressors, findings converge on the following: adverse effects in auditory fear conditioning tests persist to late adulthood for males but not females (Toledo-Rodriguez and Sandi, 2007), and performance on the MWM is significantly impaired in adulthood when the stressor occurred in adolescence as opposed to adulthood (Avital and Richter-Levin, 2005). Interestingly, chronic mild stress during the adolescent period of male rats enhanced their performance in adulthood on trace fear conditioning but did not alter performance on contextual fear conditioning, both of which are hippocampus-dependent tasks (Reich et al., 2013). Female rats who were socially isolated throughout the adolescent period as well as male and female rats who underwent chronic social instability stress during the same period, performed poorly when tested on the object recognition memory task (McCormick et al., 2010; McCormick et al., 2012; McLean et al., 2010). Remarkably, male rats undergoing chronic variable physical, but not social, stress during adolescence exhibited impaired memory performance on the MWM task during adulthood (Isgor et al., 2004). Further discrepancies in findings are observed between studies employing 5 weeks of chronic unpredictable stress during adolescence, where no memory impairment was observed when male rats were tested 10 months later (Chaby et al., 2015), as opposed to 7 weeks of chronic social stress during adolescence inducing impaired memory performance

when tested 12 months later in male mice (Sterlemann et al., 2010). Hence all in all, acute and chronic stress experiences during adolescence can incur significant long-lasting behavioural deficits, possibly driven by impaired hippocampal neurogenesis. Interestingly, social isolation seems to be one of the most critical stressors that produces robust changes in both hippocampal neurogenesis and cognitive performance (Hueston et al., 2017). Social isolation during adolescence is a highly translatable type of stressor across species, having been shown to produce similar deficits in rodents as well as in non-human primates (Cinini et al., 2014; Marlatt et al., 2011). Furthermore, isolation rearing in humans, such as neglect during early life or caregiver deprivation during adolescence, have been associated with poor cognitive control (Mueller et al., 2010). Thus, it is important to expand our understanding upon the mechanisms by which social isolation incurs its adverse effect and possible ways to revert the outcome it produces.

For humans, adolescence is a critical period of change, when one is more likely to take part in unhealthy behaviours such as alcohol and/or drug use (Kosterman et al., 2000; Martin et al., 2002), while also reduce healthy behaviours such as dynamic physical activity (Dumith et al., 2011; Nader et al., 2008). In rodent models of adolescent alcohol consumption and decreased exercise levels, impaired cognitive performance coupled with a decrease in neurogenesis has been observed (Crews et al., 2006; Crews and Nixon, 2003; van Praag, 2008). What is more, the benefits of aerobic exercise to hippocampal neurogenesis and learning in adult rodents have been well documented (van Praag, 2008; van Praag et al., 1999a; van Praag et al., 1999b). Only after a week

of involuntary exercise during adolescence, rats exhibited increased survival of proliferating cells within the DG, an increase in neuronal differentiation of these cells, as well as an increase in the transcription of the *Bdnf* gene (Lou et al., 2008). A 3-day training on a rotarod during adolescence also induced increased survival of proliferating cells (BrdU+) when measured later in adolescence in both male and female rats (DiFeo and Shors, 2017). Furthermore, forced exercise commencing at the start of the juvenile period and lasting throughout adolescence improved performance on the MWM and increased neuronal density in the DG of male rats (Uysal et al., 2005). In a similar involuntary exercise protocol employing 5 weeks of running during adolescence rather than 8, male rats who exercised outperformed their sedentary counterparts in the MWM task and also exhibited higher density of mossy fibres and BDNF protein within the hippocampus (Gomes da Silva et al., 2012). Last but not least, a recent study from our lab illustrated the differential effects of voluntary exercise when initiated during adolescence versus adulthood on the amygdala-dependent cued fear conditioning and the hippocampal-dependent context fear conditioning (O'Leary et al., 2018). Given the importance of this developmental period, it is crucial to gain more insight in the neurogenic process and regulation during adolescence.

1.10 Primary hypothesis and aims

TLX is the master regulator of adult hippocampal neurogenesis, a process that holds promise for the development of therapeutics targeting neurodegenerative diseases. The overall aim of this thesis was to investigate whether TLX is a valid drug target based on its function and mechanism of action. Our goal was to examine whether the orphan

nuclear receptor interacts with the resident immune cells in the brain (microglia) and whether external stimuli such as exercise or stress may influence the TLX-mediated transcriptional regulation of hippocampal neurogenesis. Additionally, we hypothesized that the regulatory role of TLX in the context of neurogenesis would be also reflected within a behavioural output in rodent models of TLX deficiency, which would further validate its therapeutic viability.

Aim 1: Investigate the involvement of microglia in hippocampal neurogenesis in the absence/presence of TLX. Does TLX play a role in the neuronal-microglia crosstalk?

Chapters 2 & 3

Aim2: Investigate the role of TLX in hippocampal neurogenesis during adolescence and the impact thereupon of exercise and stress.

Chapter 4

Aim 3: Investigate the role of spontaneous deletion of TLX versus targeted knockdown in the hippocampus during adolescence, on behaviour and cognitive function throughout adolescence and adulthood.

Chapter 5 & 6

Chapter 2

Absence of the neurogenesis-dependent nuclear receptor TLX induces inflammation in the hippocampus

Danka A. Kozareva[a], Cara M. Hueston[a], Ciarán S. Ó Léime[a], Suzanne Crotty[a], Peter Dockery[b], John F. Cryan[a,c] and Yvonne M. Nolan[a, c]

[a]Department of Anatomy and Neuroscience, University College Cork, Ireland

[b] Department of Anatomy, National University of Ireland, Galway, Ireland

[c] APC Microbiome Institute, University College Cork, Ireland Department, University, Cork, Ireland

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Abstract

The orphan nuclear receptor TLX (Nr2e1) is a key regulator of hippocampal neurogenesis. Impaired adult hippocampal neurogenesis has been reported in neurodegenerative and psychiatric conditions including dementia and stress-related depression. Neuroinflammation is also implicated in the neuropathology of these disorders and has been shown to negatively affect hippocampal neurogenesis. To investigate a role for TLX in hippocampal neuroinflammation, we assessed microglial activation in the hippocampus of mice with a spontaneous deletion of TLX. Results from our study suggest that a lack of TLX is implicated in deregulation of microglial phenotype and that consequently, the survival and function of newborn cells in the hippocampus is impaired. TLX may be an important target in understanding inflammatory-associated impairments in neurogenesis.

Key words: microglia; neurogenesis; hippocampus; TLX; Interleukin-1β; DCX

2.1 Introduction

The process of generating functional neurons from stem and progenitor cells in the central nervous system occurs in the adult as well as in the embryonic brain. Neurogenic niches have been identified in the adult mammalian brain including the subgranular zone (SGZ) of the hippocampus. Here, the progeny of SGZ stem cells migrate to the granule cell layer (GCL) of the dentate gyrus (DG) and integrate into hippocampal circuitry as mature excitatory neurons (Gage, 2000). Adult hippocampal neurogenesis has been implicated in learning and memory (Gould et al., 1999a; Kempermann, 2008) and has been shown to play a role in mood regulation (Balu and Lucki, 2009; O'Leary and Cryan, 2014). Moreover, decreased hippocampal neurogenesis is recognized as an important mechanism underlying cognitive deficits associated with depression as well as with normal aging (Kuhn et al., 1996; Lazarov and Marr, 2010; Marlatt and Lucassen, 2010; Spalding et al., 2013). The precise mechanisms underlying the impairment of hippocampal neurogenesis and associated cognitive impairment are not yet fully understood, however, a role for a number of intrinsic factors have been proposed (Green and Nolan, 2014; O'Leary et al., 2016b; Qu and Shi, 2009).

One such intrinsic factor is the orphan nuclear receptor subfamily 2 group E member 1 (NR2E1), also known as TLX. The alignment of the TLX gene is highly conserved across species (97% homology between mouse and human; Jackson et al., 1998). Expression of TLX is restricted to the neurogenic niches of the brain as well as the retina (Monaghan et al., 1997; Shi et al., 2004) where it has been shown to be crucial

for neural and retinal development (Li et al., 2008b; Miyawaki et al., 2004). Specifically, TLX is responsible for the timing of neurogenesis by regulating the proliferation, differentiation and migration of stem cells (Roy et al., 2004) and has been termed a master regulator of neurogenesis (Islam and Zhang, 2015). Moreover, targeted disruption and conditional deletion of TLX in mouse models have implicated TLX as an important factor in the control and maintenance of adult hippocampal neurogenesis (Murai et al., 2014; Niu et al., 2011; Roy et al., 2004) and in hippocampal neurogenesis-associated behavioural tasks in rodents such as Morris Water Maze and contextual fear conditioning (Murai et al., 2014; Zhang et al., 2008). In a spontaneous deletion mouse model, adult TLX knock out mice displayed altered neurogenesis, synaptic plasticity, and an impairment of dendritic structures in the dentate gyrus. These mice also presented with impairments in motor, cognitive and anxiety-related behaviours (Christie et al., 2006; Young et al., 2002). Mice with constitutive knock out of the TLX gene present with lower body weight, hypoplasia and distortion of the anterior aspects of the brain. In addition, the $Nr2e1^{-/-}$ mice exhibited thin and frail rostral commissure, the cerebrum and olfactory lobes were underdeveloped and the retina suffered diminished vascularization and displacement of retinal ganglion cells and optic nerves (Young et al., 2002).

Microglial cells and pro-inflammatory cytokines in the microenvironment of the subgranular neurogenic niche have also been shown to impact upon neurogenesis (Green and Nolan, 2014; Yirmiya and Goshen, 2011). Microglia are the resident immune cells of the central nervous system and have been shown to regulate adult

hippocampal neurogenesis under physiological conditions (Sierra et al., 2014a). Quiescent (also known as ramified or resting) microglia display small cell bodies and multiple extending processes, with which they probe the environment for signals of injury, trauma or other disruption of homeostasis. However, under inflammatory conditions, microglia become activated and can suppress neurogenesis (Belarbi and Rosi, 2013). Activated microglia become motile using amoeboid-like movements and display enlarged cell soma and one or less extending process (Kettenmann and Verkhratsky, 2011; Kohman and Rhodes, 2013). Activated microglia have been described as either adopting a pro- (M1) or an alternatively activated-(M2) inflammatory phenotype (Orihuela et al., 2016). Pro-inflammatory or M1 microglia are phagocytic and release inflammatory molecules including cytokines, nitric oxide and other reactive oxygen species which have been shown to have a negative effect on hippocampal neurogenesis (Belarbi et al., 2012; Ekdahl et al., 2003; Fujioka and Akema, 2010). Two such cytokines shown to be released by activated microglia and have detrimental effect on the brain are interleukin -1 beta (IL-1β) and tumour necrosis factor alpha (TNFα) (Nakamura et al., 1999; Wang et al., 2015). We have previously shown that IL-1β treatment decreases TLX expression in neurosphere cultures prepared from both embryonic and adult rat hippocampus in a time- and dose-dependent manner (Green and Nolan, 2012b; Ryan et al., 2013). Alternatively activated (M2) microglia, on the other hand, have been shown to exhibit neuroprotective properties (Orihuela et al., 2016) by releasing growth factors, enzymes and cytokines which facilitate repair and neurite outgrowth (Butovsky et al., 2006; Cherry et al., 2014). For instance, the enzyme Arginase 1 (Arg1) is released in response to wound healing and extracellular matrix deposition (Cherry et al., 2014),

while the expression of C-Type Mannose Receptor 1 (CD206) is promoted in response to stimulation by the anti-inflammatory cytokine IL-4 (Roszer, 2015). It should be noted that it has been suggested that describing activated microglia as M1 or M2 type can be restrictive and can hinder our understanding of the complex pathways via which microglia influence the brain parenchyma (Ransohoff, 2016).

The role of TLX in the activation status of microglia in the hippocampal neurogenic niche remains largely unexplored. Thus, the aim of the present study was to investigate microglial phenotypes and the hippocampal architecture in heterozygous and homozygous mice with spontaneous deletion of TLX.

2.2 Material and Methods

2.2.1 Animals

Two-month-old male $Nr2e1^{-/-}$ knockout mice, $Nr2e1^{+/-}$ heterozygous mice, and wildtype controls (129S1/SvImJ background) were group housed under standard housing conditions (temperature 21°C and relative humidity 55%), with food and water available *ad libitum*. Breeding pairs were kindly provided by Prof. Elizabeth Simpson, University of British Colombia. $Nr2e1^{-/-}$ mice exhibit a spontaneous deletion of the entire TLX allele, including all nine exons. However, the deletion of TLX does not affect the transcription of neighbouring genes (Kumar et al., 2004). All experiments were conducted in accordance with the European Directive 2010/63/EU,

and under an authorization issued by the Health Products Regulatory Authority Ireland and approved by the Animal Ethics Committee of University College Cork.

2.2.2 BrdU administration and tissue preparation

Bromodeoxyuridine (BrdU; Sigma) was administered (4 x intraperitoneal (i.p.) injections over the course of six hours at 75µg/10mL/kg) to one cohort of $Nr2e1^{-/-}$, $Nr2e1^{+/-}$ and wildtype mice at postnatal day (P) 42. Since the effect of silencing and overexpressing TLX on neuronal survival has been thoroughly examined at 3- and 4-week time points (Murai et al., 2014; Niu et al., 2011; Roy et al., 2004) we investigated the effect of TLX knock out on neuronal and cellular survival 2 weeks post BrdU injection. This allowed us to further examine whether a lack of TLX interferes at an intermittent point (2 weeks) of the neurogenic process, rather than when adult-born neurons integrate into hippocampal circuitry (4 weeks). At P56 mice were euthanized with an i.p. injection of anaesthetic (Pentobarbital; 1.0 mL/kg) and transcardially perfused using a 0.9% phosphate buffered saline (PBS) solution followed by 4.0% paraformaldehyde (PFA) in PBS. After overnight incubation in PFA, brains were incubated in 30% sucrose until they sank, and subsequently flash frozen using liquid nitrogen. Coronal sections (40 µm) through the hippocampus were collected directly onto slides in a 1:6 series, then stored at -80°C.

2.2.3 Immunohistochemistry

To determine the survival of adult-born hippocampal neurons, sections were double-labelled with BrdU and the neuronal marker NeuN. DNA was denatured in sections by incubation in 2M HCl for 45 min at 37°C, renatured in 0.1M sodium tetraborate

(pH 8.5) and then blocked in 3% normal donkey serum (NDS; Sigma D9663). Slides were incubated with anti-BrdU antibody (Abcam AB6326; 1:250) then incubated with AlexaFluor594 donkey anti-rat (Abcam Cat# ab150156; 1:500) and with NeuN (Millipore MAB377; 1:100). Finally, sections were incubated with AlexaFluor488 donkey anti-mouse (Abcam Cat# ab150105; 1:500), washed and coverslipped using anti-fade mounting medium (Dako; Cat# S3023).

The number of microglia and newly born neurons in the hippocampus was assessed by staining for their respective markers: ionized calcium binding adaptor molecule 1 (Iba-1) for microglia and doublecortin (DCX) for newly born neurons. To determine whether TLX was expressed on microglia, double-labelling was performed for TLX and Iba-1. Astrocytic activation was assessed using an antibody raised against the glial fibrillary acidic protein (GFAP). Sections were washed, incubated in 3% NDS and then in anti- Iba-1 (Wako 019-19741; 1:1000) or anti-DCX (Santa Cruz Biotechnology; Z0334; 1:100), the combination of TLX (Abcam ab86276; 1:100) and Iba1 (Abcam ab5076; 1:100), or in GFAP (DAKO z0334; 1:500). Sections were incubated in AlexaFluor488 donkey anti-rabbit (GFAP and Iba-1; Abcam ab150073; 1:500) or AlexaFluor488 donkey anti-goat (DCX; Abcam ab150129; 1:500) or the combination of AlexaFluor488 donkey anti-rabbit (TLX; Abcam ab150073; 1:500) and Alexa Fluor594 donkey anti-goat (Iba1; Abcam ab150136; 1:500) and were subsequently counterstained with DAPI (Sigma D9642; 1:5000), and coverslipped using anti-fade mounting medium (Dako; S3023).

For haemotoxylin and eosin (H&E) staining, slides were washed in distilled water incubated in haemotoxylin washed and incubated in eosin. Slides were dehydrated in a series of alcohol concentrations 70%, 90%, 95% and 100%, followed by incubations in histolene.

2.2.4 Image analysis and cell quantification

Images were obtained using either an Olympus VS120 virtual slide scanning system (courtesy of Prof. Peter Dockery, Department of Anatomy, NUI Galway, Ireland) or an Olympus FV1000 scanning laser confocal system (BioSciences Imaging Centre, Department of Anatomy and Neuroscience, University College Cork, Ireland). Z-stack images with a 1.10 μm or 4.4 μm step size were collected using a 10x objective (BrdU/NeuN), 20x objective (DCX/DAPI; Iba1/DAPI) or 40X objective (GFAP), respectively. The DG was imaged bilaterally on all sections. For bright field images an Olympus BX533 upright microscope coupled to an Olympus DP72 camera was used with 10X and 40X objectives.

Cell quantification, area and volume measurements were performed using the image processing software packages OlyVIA and ImageJ (National Institute of Health, USA; Schneider et al., 2012). Quantification of BrdU- and BrdU/NeuN- positive cells was performed for the GCL, SGZ, and the hilus of the DG. Quantification of Iba-1-positive cells was performed for the GCL and hilus of the DG. The DCX-expressing cell bodies only emerge in the SGZ and were thus quantified within this area. Systematic random sampling was employed for all cell quantifications, the unbiased Physical Paired

Dissector method (Mayhew, 1992) was performed for unbiased stereological estimation of the number of each immunopositive cell phenotype (Crotty et al., 2008). For area quantification, the area of interest, i.e. subareas of the dentate gyrus or the microglia soma, was outlined manually and its area calculated using the image processing software ImageJ. For analysis of the microglial phenotypes, Iba1+ cells with soma area equal to or below one standard deviation above the mean and more than two visible processes were categorized as ramified or resting, while cells with enlarged soma area (greater than one standard deviation above the mean) and up to one extending process were categorized as activated. The morphology of DCX+ cells was analysed with NeuronJ, an ImageJ plug-in which facilitates the tracing and quantification of elongated image structures (Meijering, 2010; Meijering et al., 2004). Specifically, the dendritic length, number of dendrites and number of nodes for 10 randomly selected cells through the DG from each of five animals per group were compared across all three groups.

2.2.5 Quantitative RT-PCR analysis of hippocampal tissue

Fresh brains from wildtype, Nr2e1$^{+/-}$, and Nr2e1$^{-/-}$ were snap frozen, and stored at -80^{o}C. The hippocampal region was micro-punched according to visual comparison to the mouse brain atlas (Franklin, 2008).

Samples were processed according to the GenElute kit protocol (Sigma; RTN350). Briefly, total cellular RNA was homogenized into lysis solution and homogenised sample was filtered through a binding column to remove non-RNA from the sample.

Equal volume of 70% ethanol was added to the filtrate and purified through columns, which were then washed with buffer. Purified mRNA was recovered into 30 µL of elution solution. A further DNAse wipeout step was conducted on the sample using DNase1 (Sigma; AMPD1) to ensure the complete removal of endogenous DNA from the samples. Total RNA yield and purity were determined using the Nanodrop System (Thermo Scientific). Synthesis of cDNA was performed using 0.5 µg of normalized total RNA from each sample using ReadyScript cDNA synthesis mix (Sigma; RDRT-25RXN). Probed cDNA amplification was performed in a 20 µL reaction consisting of 10 µL KiCqStart qPCR Ready Mix with ROX (Sigma; Cat# KCQS02), 0.1 µL of each forward and reverse primer (final concentration 250 nM), 1 µL cDNA template, and 8.8 µL RNase-free water. Real-time RT PCR was performed in duplicate in a 96-well plate (Applied Biosystems) and captured in real time using the StepOne Plus System (Applied Biosystems). Relative gene expression was adjusted to the housekeeper gene *Trfc*, and quantified using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Primer sequences for the detection were: 5`-CCCAAGTATTCTCAGATATGATTTCAA-3` (forward) and 5`-AAAGGTATCCCTCCAACCACTC-3` (reverse) for *Trfc*; 5`-TGCCACCTTTTGACAGTGATG-3` (forward) and 5`-TGATGTGCTGCTGCGAGATT-3` (reverse) for *IL-1β*; 5`-AGGCACTCCCCCAAAAGATG-3` (forward) and 5`-TTGCTACGACGTGGGCTAC-3` (reverse) for *TNFα*; 5` -TGGGTGGATGCTCACACTG- 3` (forward) and 5`-ACAGGTTGCCCATGCAGATT-3` (reverse) for *Arg1*; and 5` -

GTGGGGACCTGGCAAGTATC-3` (forward) and 5`-CACTGGGGTTCCATCACTCC-3' (reverse) for *CD206*.

2.2.6 Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA) with an α-level of 0.05. In cases where data were not normally distributed or the assumption of homogeneity of variance was violated, the non-parametric Kruskal-Wallis analysis of variance by ranks was performed. Posthoc analyses were conducted using either Bonferroni's multiple comparisons or the Mann-Whitney multiple comparisons test for parametric and non-parametric data, respectively. All data are presented as mean ± SEM.

2.3 Results

2.3.1 Impaired hippocampal architecture and reduced neurogenesis and survival of adult born cells in Nr2e1$^{-/-}$ but not Nr2e1$^{+/-}$ mice

Histological examination of coronal sections through the hippocampus of Nr2e1$^{-/-}$ mice identified distortion of the hippocampal structure compared with wildtype and Nr2e1$^{+/-}$ littermate controls (See Figure 2.1). The vulnerable nature of the tissue from Nr2e1$^{-/-}$ mice resulted in the appearance of vacuoles in the tissue. Fluorescent labelling with the nuclear stain DAPI illuminated the gross impairments in the structure of the DG of Nr2e1$^{-/-}$ mice that were also apparent in the H&E-stained sections. While no

differences were evident between the dentate gyri of mice heterozygous for TLX and their wildtype littermates, the animals lacking TLX exhibited a notably smaller dentate structure with a significantly altered shape. Specifically, the upper and lower blades of the DG were both shorter and the hilus appeared wider in the Nr2e1$^{-/-}$ mice (See Figure 2.1).

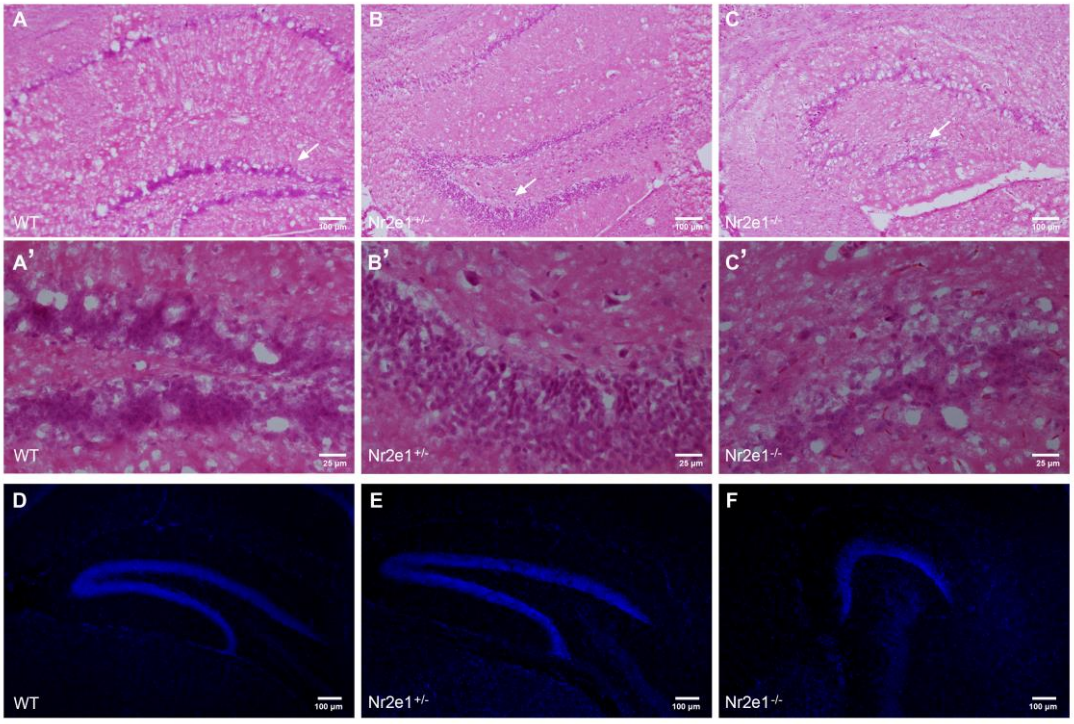

Figure 2.1: Representative images through coronal sections of the hippocampal formation showing low and high magnification of H&E staining in the DG of wildtype **(A**: 10X; **A'**: 40X**)**, Nr2e1$^{+/-}$ **(B**: 10X; **B'**: 40X**)**, and Nr2e1$^{-/-}$ **(C**: 10X; **C'**: 40X**)** mice. Representative images of nuclear staining (DAPI) in the DG in wildtype **(D)**, Nr2e1$^{+/-}$ **(E)**, and Nr2e1$^{-/-}$ **(F)** mice. Scale bar **(A-C & D-F)** = 100 µm. Scale bar **(A'-C')** = 25 µm.

We observed a significant decrease in the number of surviving adult-born cells (BrdU+) in the DG of Nr2e1$^{-/-}$ mice compared to either wildtype or Nr2e1$^{+/-}$ mice ($F(2, 10) = 6.354$; $p < 0.001$). This difference was driven by a reduction of BrdU+ cells in the SGZ of Nr2e1$^{-/-}$ mice ($F(2, 10) = 5.115$; $p < 0.05$) compared to their wildtype and heterozygous littermates; no significant change was observed across the three genotypes in the GCL ($F(2, 10) = 0.159$; $p > 0.05$) or the hilus ($F(2, 10) = 1.285$; $p >$

0.05; Figure 2.2). There was a significant decrease in the mean number of surviving new neurons in the DG of Nr2e1$^{-/-}$ compared to wildtype mice (H (2) = 6.12; $p < 0.05$). Additionally, the number of surviving neurons in the SGZ was lower in Nr2e1$^{-/-}$ compared to wildtype and heterozygous littermates (H (2) = 7.71; $p < 0.05$). There was no significant effect of genotype in either the GCL (H (2) = 1.19, p = .55) or the hilus (H (2) = .08, p = .96; Figure 2.2).

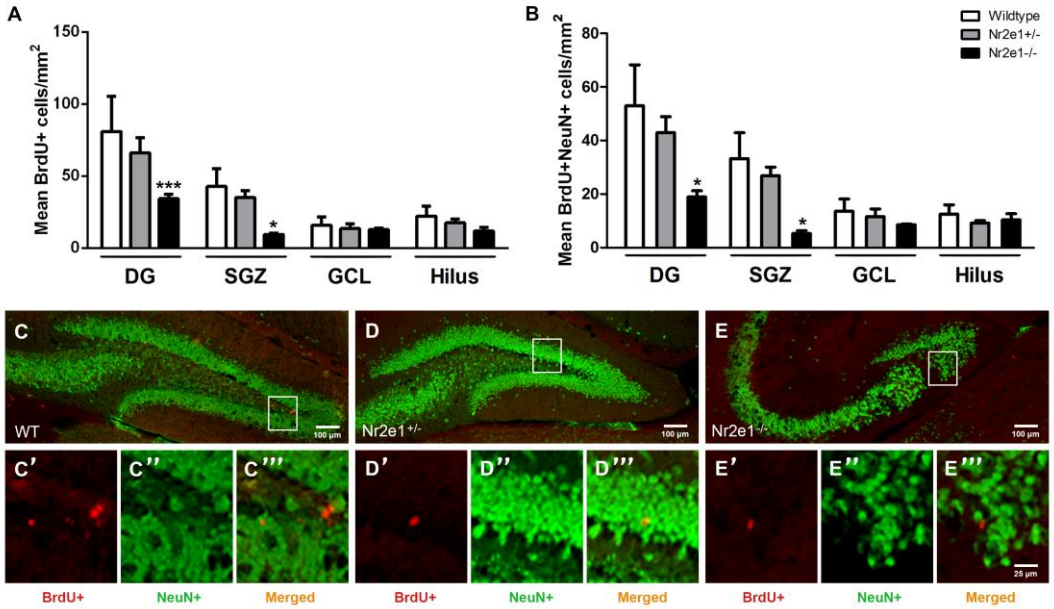

Figure 2.2: Lack of TLX causes a reduction in cell survival in the DG of Nr2e1-/- but not Nr2e1+/- mice. Mean density (cells/mm2) of adult-born surviving cells (BrdU+; A) and adult-born surviving neurons (BrdU+NeuN+; B) in the DG, SGZ, GCL and hilus of wildtype, Nr2e1+/-, and Nr2e1-/- mice. Data are expressed as mean ± SEM. *** $p < 0.001$, * $p < 0.05$ compared to wildtype and Nr2e1+/- (ANOVA), n = 4-5. Representative confocal images from wildtype (C), Nr2e1+/- (D), and Nr2e1-/- (E) of coronal sections through the DG immunohistochemically stained with BrdU (red), NeuN (green) and BrdU+NeuN+ (orange) at 10X magnification. Scale bar = 100 µm. Higher magnification images depict immunopositive cells in the DG of wildtype (C': BrdU+ channel; C'': NeuN channel; C''': merged channel), Nr2e1+/- (D': BrdU+ channel; D'': NeuN channel; D''': merged channel) and Nr2e1-/- (E': BrdU+ channel; E'': NeuN channel; E''': merged channel). Scale bar = 25 µm.

There was a significant decrease in the number of DCX+ cells in Nr2e1$^{-/-}$ mice (F (2, 9) = 22.65; $p < 0.001$) compared to both wildtype and Nr2e1$^{+/-}$ animals (Figure 2.3). DCX-positive cells which extended processes through the GCL were evident in wildtype and Nr2e1$^{+/-}$, but not in Nr2e1$^{-/-}$ mice (Figure 2.3). Morphological analysis

of DCX+ cells revealed that Nr2e1$^{-/-}$ mice exhibited a significant decrease in dendritic length ($F(2, 27) = 8.525$; $p < 0.01$; Figure 2.3), a reduced number of dendrites ($F(2, 27) = 4.219$; $p < 0.05$; Figure 2.3) and a diminished number of branching points of the dendritic tree ($F(2, 27) = 4.643$; $p < 0.05$; Figure 2.3) compared to wildtype control mice.

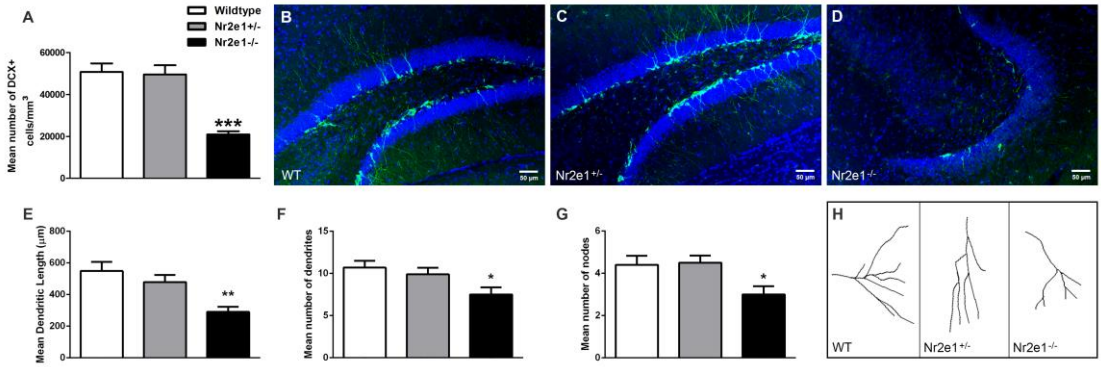

Figure 2.3: Lack of TLX is associated with reduction in neurogenesis and impaired morphology of newly born neurons in the DG of Nr2e1$^{-/-}$ but not Nr2e1$^{+/-}$ mice.
Mean number of DCX+ cells per mm^3 in wildtype (WT), Nr2e1$^{+/-}$, and Nr2e1$^{-/-}$ animals (A), Representative confocal images through the DG of DCX+ (green) cells in wildtype (B), Nr2e1$^{+/-}$ (C), and Nr2e1$^{-/-}$ (D) mice. Nuclei were counterstained with DAPI (blue). Scale bar = 50 µm. Mean dendritic length (µm) of DCX+ cells (E), mean number of dendrites per DCX+ cell (F) and mean number of nodes per DCX+ cell (G) in wildtype, Nr2e1$^{+/-}$, and Nr2e1$^{-/-}$ mice. Data are expressed as mean ± SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared to wildtype and Nr2e1$^{+/-}$ (ANOVA), n = 4-5. Representative tracings of cells from each group (H).

2.3.2 Microglia quantification and phenotype characterization

There was a significant increase in number of microglia in the DG and GCL of Nr2e1$^{-/-}$ mice compared to either their wildtype or Nr2e1$^{+/-}$ littermates (DG: $F(2, 9) = 16.43$, $p < 0.001$; GCL: $F(2, 9) = 30.51$, $p < 0.001$). There was no difference in microglia density in the hilus ($F(2, 9) = 1.435$; $p = 0.28$) across the three genotypes (Figure 2.4). Mice lacking TLX displayed microglia with greater cell somal area in the DG and GCL compared to wildtype or Nr2e1$^{+/-}$ mice (DG: $F(2, 9) = 5.387$; $p < 0.05$; GCL: $F(2, 9) = 7.665$; $p = 0.01$; Figure 2.4). Within the hilus we did not observe any

differences in microglia size across the three groups ($F(2, 9) = 2.634$; $p = 0.13$; Figure 2.4). The proportion of "resting" or ramified microglia in the DG of mice with complete deletion of the TLX gene was significantly smaller than the proportion of ramified microglia in the wildtype controls ($F(2, 9) = 5.683$; $p < 0.05$). The same pattern was observed in the GCL, but not the hilus (GCL: $F(2, 9) = 5.480$; $p < 0.05$; Hilus: $F(2, 9) = 3.245$; $p = 0.09$; Figure 2.4). Finally, when we compared the proportion of activated microglia (having enlarged soma) across the three genotypes we found a significant increase of activated microglia in $Nr2e1^{-/-}$ mice compared to their wildtype and $Nr2e1^{+/-}$ littermates across all areas examined (DG: $F(2, 9) = 6.531$; $p = 0.01$; GCL: $F(2, 9) = 5.711$; $p < 0.05$; Hilus: $F(2, 9) = 4.119$; $p = 0.05$; Figure 2.4).

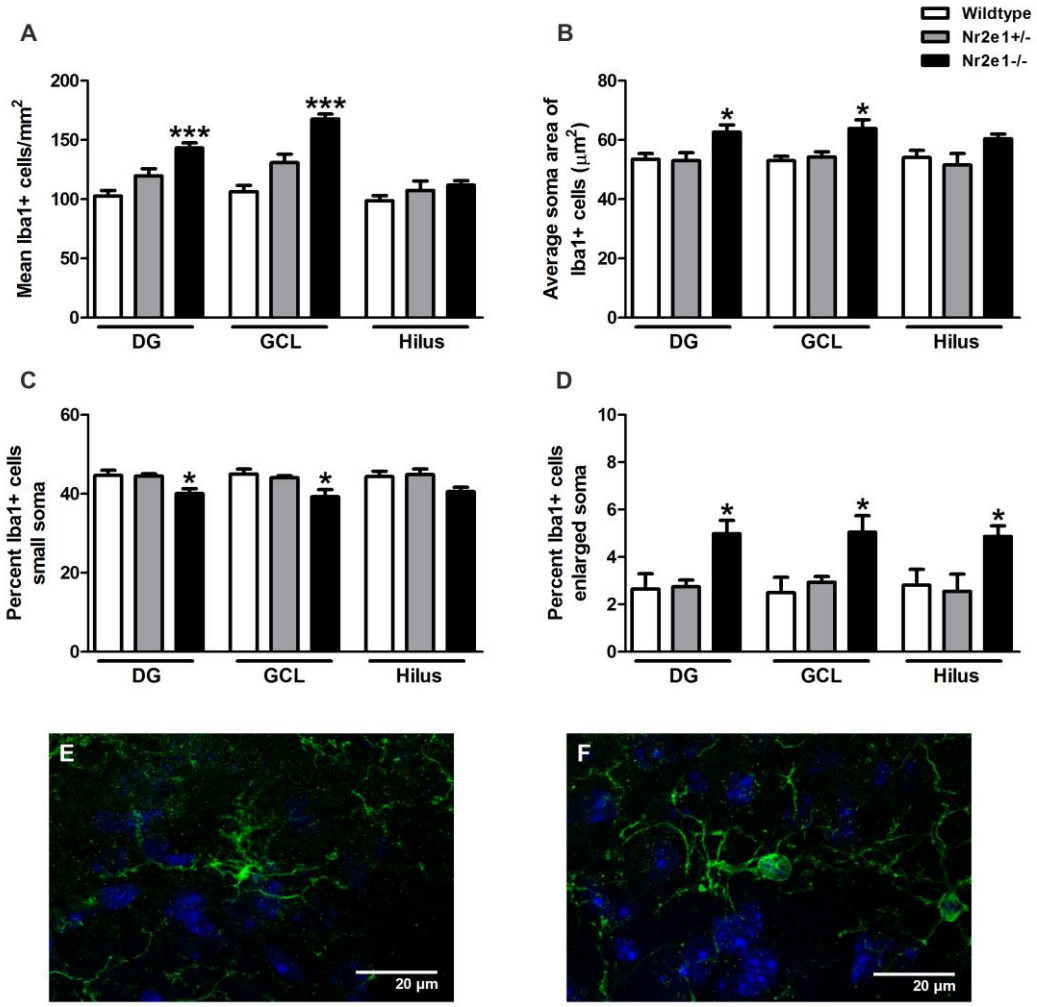

Figure 2.4: Lack of TLX is associated with increased microglia density and activation.
The mean density (cells/mm^2) of microglia (Iba1+) in the DG, GCL and hilus of wildtype (WT), Nr2e1$^{+/-}$, and Nr2e1$^{-/-}$ mice (A). Average microglial soma area (μm^2) in the DG, GCL and hilus of wildtype (WT), Nr2e1$^{+/-}$, and Nr2e1$^{-/-}$ mice (B). Proportion (%) of "resting" (C) and activated (D) microglia in the DG, GCL and hilus of wildtype (WT), Nr2e1$^{+/-}$, and Nr2e1$^{-/-}$ mice. Data are expressed as mean ± SEM. *** $p < 0.001$, * $p < 0.01$ compared to wildtype and Nr2e1$^{+/-}$ (ANOVA), $n = 4$. Representative images of a ramified/resting Iba1+ cell (E) and activated Iba1+ cell (F) at 100X magnification.

There was a significant increase in the relative mRNA expression of the pro-inflammatory cytokine IL-1β in the hippocampus of mice lacking the TLX gene ($F(2, 15) = 17.79$; $p < 0.001$; Figure 2.5). There was no difference in relative mRNA expression of TNFα across the groups ($F(2, 17) = 0.295$; $p = 0.75$; Figure 2.5). Lastly, when we compared the relative mRNA expression of the alternatively activated

markers Arg1 and CD206, we observed no difference across the three genotypes (Arg1: F (2, 21) = 0.174; p > 0.05; Figure 2.5; CD206: F (2, 21) = 0.252; p > 0.05; Figure 2.5).

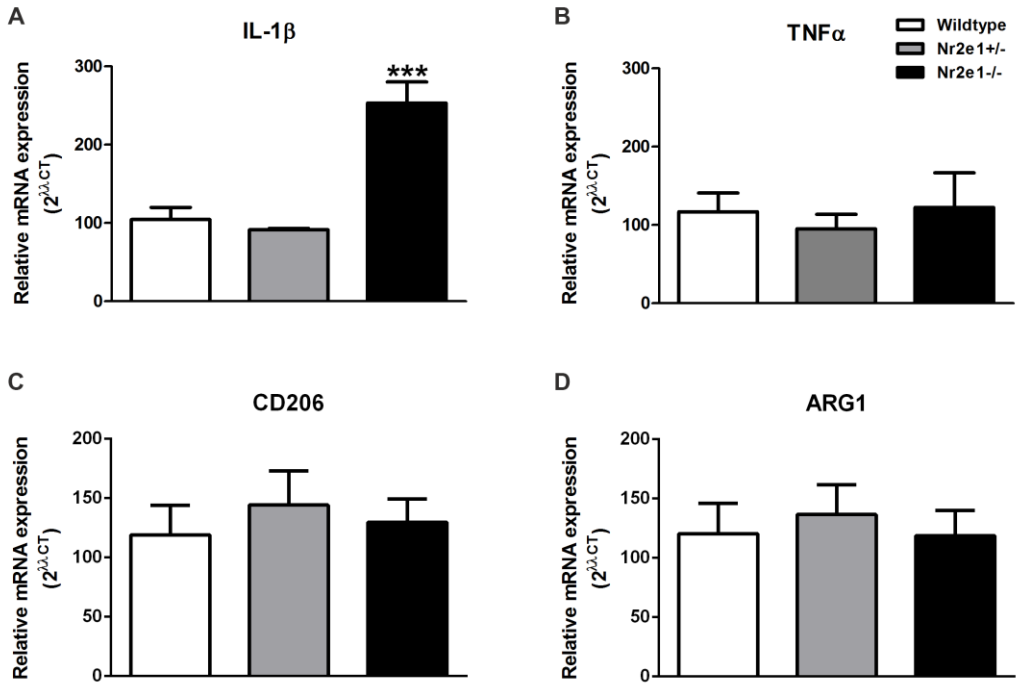

Figure 2.5: Lack of TLX is associated with increase in pro- but not anti- inflammatory markers. Relative mRNA expression of the pro-inflammatory cytokines IL-1β (A) and TNFα (B) and the anti-inflammatory markers CD206 (C) and Arg1 (D) in the hippocampus of wildtype, $Nr2e1^{+/-}$ and $Nr2e1^{-/-}$ mice. All values were adjusted to the relative expression of the housekeeping gene *Trfc*. Data are expressed as the mean ± SEM, *** $p < 0.001$ (ANOVA), $n = 7$-9.

We previously confirmed the expression of TLX in astrocytes derived from cultures of hippocampal neural stem cells (Green and Nolan, 2012b). Here we show that immunostaining of hippocampal tissue from wildtype mice revealed no overlap between TLX and Iba-1 expression, (Figure 2.6). Upon examination of GFAP staining, astrocytes appeared to have adopted a reactive or activated phenotype in the hippocampus of $Nr2e1^{-/-}$ mice when compared to $Nr2e1^{+/-}$ and wildtype mice as evidenced by increased fluorescence intensity and hypertrophy of processes (Figure 2.6).

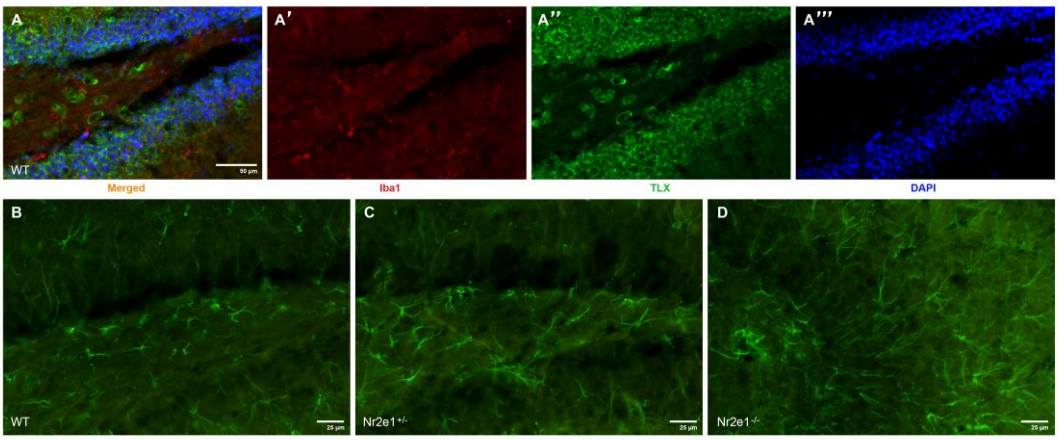

Figure 2.6: Lack of TLX expression on microglia and increase in GFAP staining in the absence of TLX. Representative image of the hippocampus of a wildtype mouse showing lack of colocalizationn between TLX and Iba1 by double immunofluorescence (A). Iba1+ staining appears as red (A'), TLX staining appears as green (A'') and nuclei were counterstained with DAPI (blue; A'''). Scale bar = 50 μm. Fluorescence images of astrocytes stained for GFAP (green) in wildtype (B), Nr2e1$^{+/-}$ (C), and Nr2e1$^{-/-}$ (D) mice. Images were taken at 40X magnification. Scale bar = 25 μm.

2.4 Discussion

In the present study, we demonstrated a pro-inflammatory phenotype in the DG of Nr2e1$^{-/-}$ mice. Specifically, we observed a significant increase in density of endogenous microglia in the dentate area of Nr2e1$^{-/-}$ mice, while Nr2e1$^{+/-}$ and wildtype mice displayed similar and lower densities of Iba1+ cells. We found that TLX deficient animals exhibited a higher percentage of microglia that were in an activated state and reduced percentage of ramified microglia. Furthermore, there was a significant increase in expression of the pro-inflammatory cytokine IL-1β, an indicator of classically activated microglia in the hippocampus of Nr2e1$^{-/-}$ mice, but no difference in the expression levels of TNFα or the alternatively activated markers CD206 and Arg1. These results were coupled with deficits in neuronal morphology and neurogenesis in Nr2e1$^{-/-}$ but not Nr2e1$^{+/-}$ mice as shown by a reduction in the density, dendritic length and nodes of newborn neurons as well as the density of surviving new neuronal and non-neuronal cells in the DG.

The current results support previous work by Monaghan and colleagues (1997) who showed alteration in the size and architecture of the hippocampus of $Nr2e1^{-/-}$ (in which the gene was functionally knocked down through homologous recombination) compared to wildtype mice (Monaghan et al., 1997). We also showed that the hippocampal architecture of the $Nr2e1^{+/-}$ mice resembles that of wildtype mice. The decrease in the number of adult born neurons in transgenic mice with a targeted deletion of TLX was first described by Shi and colleagues in 2004. Later the Simpson group showed that mice with spontaneous deletion of TLX not only displayed a decrease in neurogenesis, but also an impairment in synaptic plasticity and in the dendritic structure of pyramidal cells in the dentate gyrus but not in the CA1 region of the hippocampus (Christie et al., 2006). Here we further demonstrated that mice with a spontaneous deletion of TLX also have reduced survival of newborn neurons (BrdU+/NeuN+) as well as newborn non-neuronal cells (BrdU+) in the DG as well as impaired morphology of newly born neurons. These findings support previous evidence from studies employing mice with a conditional deletion of TLX that reported that ablation of TLX resulted in a complete loss of transiently amplifying cells and neuroblasts (Niu et al., 2011; Roy et al., 2004). The current results also suggest that a lack of the TLX gene has long term negative consequences for the viability of both neurogenic and gliogenic cells. To the best of our knowledge, we are the first to demonstrate that $Nr2e1^{+/-}$ mice do not exhibit the neurogenic and neuronal survival deficiencies observed in $Nr2e1^{-/-}$ mice. Interestingly, we have previously observed that these $Nr2e1^{+/-}$ heterozygous mice performed similarly to wildtype littermates in contextual fear conditioning, a neurogenesis-associated hippocampal-

dependant cognitive task, while Nr2e1$^{-/-}$ mice displayed impaired performance (O'Leary et al., 2016a). Together, these findings suggest that one allele of the TLX gene is sufficient for normal hippocampal neurogenic processes and associated behaviours to occur. This is in line with the proposition that an animal with a heterozygous genotype generally has a higher relative fitness than an animal having a homozygous dominant or homozygous recessive genotype (Charlesworth and Willis, 2009). Indeed, studies have shown, that next to being the master regulator of neural stem cell maintenance (Islam and Zhang, 2015), TLX is also responsible for gliomagenesis in the adult neurogenic niches (Zou et al., 2012). What is more, increased TLX expression in stem cells from gliomas correlated with poor survival of patients (Park et al., 2010b), while downregulation of TLX was shown to inhibit tumorigenesis (Cui et al., 2016; Xie et al., 2014). Hence, one allele of the TLX gene may not only be sufficient but also beneficial from an evolutionary perspective.

Interestingly, mice with a spontaneous deletion of TLX did not exhibit full ablation of cell survival and neurogenesis as evidenced by a third of BrdU+, BrdU+NeuN+ and DCX+ cells remaining in the DG. This could be explained by the fact that neurogenesis is regulated by multiple pathways operating in parallel and/or in synergy that could compensate for the lack of TLX. For example, the Wnt/beta-catenin pathway (Qu et al., 2010), Notch signalling (Ables et al., 2010; Breunig et al., 2007) and the sonic hedgehog (Shh) pathway (Ahn and Joyner, 2005; Machold et al., 2003) have been identified as central mediators of NSC maintenance. Indeed, TLX has been shown to directly activate both the Wnt/beta-catenin (Qu et al., 2010) as well as the Notch

signalling pathway (Shi, 2015). Conversely, Shh signalling appears to take place upstream of TLX (Shimozaki et al., 2012). It is also possible that in the absence of TLX expression, other transcription factors, such as SOX2 (Shimozaki et al., 2012), autonomously maintain, albeit a reduced pool of NSCs.

Despite the reduction in size of the hippocampus and the reduced cell survival observed, we found a pattern towards an increased number of endogenous microglia in the DG and GCL of the hippocampus of Nr2e1$^{-/-}$ mice. In recent years, microglia have been shown to be important effectors of adult hippocampal neurogenesis. In the absence of inflammation, ramified microglia are involved in pruning newborn cells, in providing trophic support for newly forming cells, and in apoptosis of newborn cells that fail to integrate into the existing circuitry (Sierra et al., 2014a; Sierra et al., 2010). *In vitro* studies have also shown that in their resting state, microglia release factors which rescue neuroblasts, instruct neuronal cell differentiation and enhance and prolong the neurogenic potential of the cultured cells (Walton et al., 2006). However, microglia that become activated by foreign antigens or by changes in brain homeostasis are predominantly neurotoxic, promote an inflammatory environment and have been shown to suppress hippocampal neurogenesis by reducing the survival of neuroblasts (Ekdahl et al., 2003; Monje et al., 2003). We thus characterized the activation status of the microglia in the DG of Nr2e1$^{-/-}$ mice by firstly assessing the morphology of the cells, and observed that there was a significant increase in the proportion of activated microglia in the whole DG, including the GCL and hilus, of Nr2e1$^{-/-}$ mice. This result was coupled with a significant decrease in the proportion of

ramified Iba1+ cells in the DG of Nr2e1$^{-/-}$ animals. We showed through immunolabelling that TLX is not expressed on microglia. This finding is corroborated by RNA sequencing data (Zhang et al., 2014) and gene expression analysis and histology (Zou et al., 2012). Future studies should examine whether there is causal link between the lack of TLX expression and the increase in microglial activation that we observed. If such causality exists, it would be important to identify the underlying pathway mediating this process as potential therapeutic targets for inflammatory and/or neurodegenerative conditions may be revealed. We speculate that astrocytes may be key mediator of the activation of microglia under reduced or ablated TLX expression. In our transgenic model, we observed increased intensity of GFAP fluorescent staining and extended or hypertrophic GFAP processes in Nr2e1$^{-/-}$ mice. These parameters have been established as markers of astrocyte activation and reactive gliosis (Pekny et al., 2016; Pekny et al., 2014; Wilhelmsson et al., 2006). Furthermore, it has been shown that TLX regulates astrogenesis in the subventricular zone (SVZ) (Qin et al., 2014) as well as astrocyte development in the retina (Miyawaki et al., 2004). Additionally, loss of TLX expression in the SVZ also resulted in increased GFAP fluorescence staining and extended GFAP+ cell processes (Li et al., 2012a). Whether the spontaneous deletion of TLX in our model causes direct activation of astrocytes, which thereby activate microglia; or whether a lack of TLX results in activation of microglia, which cause astrocytes to adopt reactive phenotype remains to be investigated.

In order to determine whether the increased number of activated microglia in Nr2e1$^{-/-}$

mice display a pro-inflammatory or neuroprotective phenotype, we examined the relative mRNA expression of pro-inflammatory cytokines IL-1β, and TNFα as well as the expression of alternatively activated markers Arg1 and CD206 in the hippocampus of these mice. We found that there was a significant increase in the mRNA expression of IL-1β but not TNFα in $Nr2e1^{-/-}$ compared to wildtype and $Nr2e1^{+/-}$ mice. Furthermore, no difference was observed in the expression of the alternatively activated markers. The appearance of microglial-derived IL-1β under endotoxic conditions was first investigated several decades ago (van Dam et al., 1992) This and a related classic pioneering study implicated IL-1β as a key player in the signalling pathway between neuronal and microglial cells in the brain during inflammatory challenge (Van Dam et al., 1995; van Dam et al., 1992). Elevated levels of IL-1β have previously been shown to negatively impact upon neurogenesis. For example, central infusion of IL-1β to rats and overexpression of IL-1β in a conditional transgenic mouse model both significantly reduced neurogenesis in the DG (Goshen et al., 2008; Koo and Duman, 2008; Wu et al., 2012). *In vitro* studies have revealed that IL-1β reduced the number of proliferating cells, proliferating newly-born neurons, reduced neuronal differentiation, and stimulated astroglial differentiation in cultures prepared from adult and embryonic rat hippocampal neural precursor cells (Green and Nolan, 2012b; Green et al., 2012; Koo and Duman, 2008; Ryan et al., 2013). We have previously demonstrated the vulnerability of TLX to IL-1β in embryonic rat hippocampal neural stem cells under both proliferation and differentiation conditions (Green and Nolan, 2012b). The IL-1β-induced reduction of the numbers of proliferating cultured neural precursor cells and reduction of TLX expression was rescued by inhibition of GSK-3β signalling, which has been shown to negatively regulate neurogenesis (Green and

Nolan, 2012b). Additionally, we have previously shown that treatment with IL-1β reduced TLX expression in proliferating neural stem cells in the adult hippocampus in a dose and time-dependant manner. Further, administration of the IL-1 receptor antagonist or IL-1 receptor silencing prevented this decrease (Ryan et al., 2013). Interestingly, others have shown that astrocyte-mediated secretion of IL-1β occurs under inflammatory conditions in human astrocytes cultures (Didier et al., 2003) and spinal cord injury in rats induces increased expression of IL-1β in astrocytes located around the spinal cord ependyma, a neurogenic region (Paniagua-Torija et al., 2015). Recently it was demonstrated that reactive astrocytes are induced by activated microglia (Liddelow et al., 2017), which points to the question of whether activated astrocytes or microglia occurs first as a result of lack of TLX expression.

Overall, these studies suggest that a lack of TLX expression may be implicated in microglial and astrocytic activation which is coupled with impaired survival and integration of hippocampal newborn neurons. Moreover, increased levels of endogenous IL-1β in the hippocampus of $Nr2e1^{-/-}$ mice correlate with the observed deficiencies in hippocampal neurogenesis and increased microglia activation. Given our previous studies demonstrating that IL-1β induces a decrease in expression of TLX in hippocampal neural stem cells (Green and Nolan, 2012b), it is also likely that a vicious circle of an IL-1β-induced inflammatory environment and an impaired neurogenic phenotype is perpetuated in the absence of TLX expression. Furthermore, it would be important to establish the temporal relation between a lack of TLX

expression, astrocyte activation and microglia activation, which may aid in identifying the primary cause for the observed increase in IL-1β in $Nr2e1^{-/-}$ mice.

Chapter 3

A role for the orphan nuclear receptor TLX in the interaction between neural precursor cells and microglia

Danka A. Kozareva[a,b], Gerard M. Moloney[a], Alan E. Hoban[a], Valerio Rossini[b,c], Ken Nally[b,c], John F. Cryan[a,b], Yvonne M, Nolan[a,b]

[a] Department of Anatomy & Neuroscience, University College Cork, Ireland

[b] APC Microbiome Institute, University College Cork, Ireland

[c] School of Biochemistry and Cell Biology, University College Cork, Ireland

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Abstract

Microglia are an essential component of the neurogenic niche in the adult hippocampus and are involved in the control of neural precursor cell proliferation, differentiation and the survival and integration of newborn neurons in hippocampal circuitry. Microglial and neuronal crosstalk is mediated in part by the chemokine CX3CL1 (fractalkine) released from neurons, and its receptor CX3C chemokine receptor 1 (CX3CR1) which is expressed on microglia. A disruption in this pathway has been associated with impaired neurogenesis yet the specific molecular mechanisms by which this interaction occurs remain unclear. The orphan nuclear receptor TLX (Nr2e1) is a key regulator of hippocampal neurogenesis and we have shown that in its absence microglia exhibit a pro-inflammatory activation phenotype. However, it is unclear whether a disturbance in CX3CL1/CX3CR1 communication mediates an impairment in TLX-related pathways which may have subsequent effects on neurogenesis. To this end, we assessed microRNA (miRNA) expression of up- and down- stream signalling molecules of TLX in the hippocampus of mice lacking CX3CR1. Our results demonstrate that a lack of CX3CR1 is associated with altered expression of TLX and its downstream targets in the hippocampus without significantly affecting upstream TLX regulators. Thus, TLX may be a potential participant in neural stem cell-microglial crosstalk and may be an important target in understanding inflammatory-associated impairments in neurogenesis.

Key words: Hippocampal neurogenesis; Microglia; CX3CR1; TLX; Neural precursor cells

3.1 Introduction

Hippocampal neurogenesis, the process of generating functional new neurons from neural stem cells (NSCs), occurs throughout the lifespan in most mammalian species and plays a role in certain forms of learning, memory and in mood regulation (Cameron and Glover, 2015). The discussion on whether hippocampal neurogenesis actually occurs in the adult human brain has recently been renewed (Sorrells et al., 2018; Boldrini et al., 2018). However, the hypothesis that adult-generated neurons can make important functional contributions to neural plasticity and cognition across the lifespan in humans is still widely maintained (reviewed by Kempermann et al., 2018). Microglia are an essential component of the neurogenic niche in the adult hippocampus and provide trophic support for the neurogenic process (Gemma and Bachstetter, 2013; Eggen et al., 2013). Specifically, microglia promote the proliferation of neural precursor cells (NPCs) as well as the survival of newly born neurons through the secretion of neurotrophic factors such as insulin-like growth factor 1 (IGF-1) and brain-derived neurotrophic factor (BDNF) (Sato, 2015; Ziv and Schwartz, 2008) and play an important role in the pruning of apoptotic adult born neurons during the critical period – 1 to 4 days after cell birth. The majority of these transiently amplifying progenitors become apoptotic during the transformation to neuroblasts, and ramified microglia remove them from the neurogenic niche through phagocytosis (Sierra et al., 2010; Sierra et al., 2014b). Recent evidence shows that microglia in turn can be influenced by neuronal cells (Turano et al., 2017). However, the signalling pathways underlying NPC-microglia interaction are yet to be fully explored.

Intracellular crosstalk between neurons and microglia can occur through a variety of signalling mechanisms, one of which is through the chemokine system (Allen et al., 2007; Rossi and Zlotnik, 2000). Signalling occurs between the membrane-bound ligand fractalkine, also known as CX3CL1, which is constitutively expressed by neurons, and its cognate receptor CX3C chemokine receptor 1 (CX3CR1), which in the healthy brain is selectively found on microglia (Harrison et al., 1998; Lauro et al., 2015; Nishiyori et al., 1998). During early postnatal development, signalling between the CX3CL1/CX3CR1 pair drives synaptic pruning (Paolicelli et al., 2011), elimination of supernumerary neurons (Gemma and Bachstetter, 2013), and fine-tuning of anatomical connections to ensue correct functional maturation and cell positioning (Limatola and Ransohoff, 2014; Paolicelli et al., 2014). During adulthood, CX3CL1 is expressed in particularly high levels in hippocampal neurons (Harrison et al., 1998) and has been shown to stimulate the survival of NPCs *in vitro* (Krathwohl and Kaiser, 2004). Direct evidence for the role of CX3CL1/CX3CR1 signalling in adult hippocampal neurogenesis has recently emerged. For instance, targeted knockdown or pharmacological inhibition of CX3CR1 in adult rats resulted in a marked decrease of NPC proliferation and newborn neuron survival in the subgranular zone (SGZ) of the dentate gyrus (DG), the neurogenic niche of the hippocampus (Bachstetter et al., 2011). Additionally, it was demonstrated that the CX3CR1-GFP knock out ($CX3CR1^{KO}$) mice and their heterozygote littermates exhibit decreased hippocampal neurogenesis and survival in a gene-dose dependent manner, which was coupled with reduced synaptic plasticity and impaired performance in neurogenesis-associated tasks, such as contextual fear conditioning and learning in the Morris water

maze task (Rogers et al., 2011). Employing immunohistochemistry and unbiased stereology methods, the authors showed that in the absence of CX3CR1 there was a significant decrease in the number of doublecortin (DCX+) expressing newborn neurons, a marker of neurogenesis. CX3CR1 knockout mice also exhibited reduced proliferation as evidenced by a significantly lower number of cells incorporating the thymidine analogue BrdU 24 hours post injection. Indeed, CX3CR1 deficient mice were found to present with impaired hippocampal neurogenesis, not only during adulthood, but also during late adolescence/early adulthood as well as during aging (Vukovic et al., 2012). Furthermore, using the same model it has been shown that CX3CL1/CX3CR1 signalling is involved in adult hippocampal, but not olfactory bulb neurogenesis (Reshef et al., 2014). Corroborating these results, another study demonstrated that the decrease of hippocampal neurogenesis in the absence of CX3CR1 expression, as measured by reduced number of DCX+ cells in the hippocampus of CX3CR1 knockout mice compared to their heterozygous littermates, was coupled with reduced dendritic complexity and delayed maturation of the newborn neurons (Xiao et al., 2015). This finding illustrates that CX3CR1/CX3CL1 has a role to play in dendritic development and maturation from new neurons and thus in neural signal integration into the neuronal circuitry. It is thus important to recognize that the lack of CX3CR1 in the adult hippocampus has detrimental effects on the multiple stages of the neurogenic process from proliferation to survival and maturation (Rogers et al., 2011; Xiao et al., 2015; Sellner et al., 2016). It has also been shown that the reduced number of DCX+ cells observed in the SGZ of $CX3CR1^{KO}$ mice was specific to knockout of the receptor, as mice that exhibited CX3CL1 knockout did not produce the same deficit (Sellner et al., 2016). Moreover, the authors of this report

showed that the CX3CR1 knockout mice exhibited a consistent reduction in the number of BrdU+, BrdU+DCX+, BrdU+NeuN+ as well as DCX+ and NeuN+ cells without displaying a reduction in total hippocampal volume (Sellner et al., 2016). Collectively, these data position the CX3CR1/CX3CL1 pathway as a major mediator of hippocampal neurogenesis.

The orphan nuclear receptor TLX (*Nr2e1*) whose expression is confined to stem cells in the neurogenic niches of the adult brain is a key intrinsic regulator of hippocampal neurogenesis (Islam and Zhang, 2015) and it exercises this role by regulating a number of different genes and pathways. For instance, it is a transcriptional repressor and regulates the expression of genes involved in multiple pathways important for the generation of neurons such as cell adhesion (Sun et al., 2007), DNA replication (Liu et al., 2010b) and cell cycle (Niu et al., 2011). As a transcriptional repressor, TLX targets genes such as the cyclin dependent kinase inhibitor p21 and the tumour suppressor gene pten thus promoting NSCs proliferation (Li et al., 2008b; Niu et al., 2011; O'Leime et al., 2017; Sun et al., 2007), as well as the member of the bone morphogenetic protein family bmp4 resulting in the inhibition of NSC astrocytic differentiation (Qin et al., 2014). TLX can also bind to its own promoter thereby suppressing its transcriptional activity through a feedback loop, which can be antagonized by the SRY-box-containing Gene 2 (Sox2), which also binds to an upstream region of the TLX gene. What is more, it has been shown *in vitro* that TLX and Sox2 interact physically, whereby Sox2 acts as a transcriptional activator of TLX, to promote NSC maintenance and self-renewal (Shimozaki et al., 2012). Multiple

small non-coding RNAs are differentially expressed in the hippocampus and a subgroup of them (microRNAs) has been shown to fine-tune the progression of adult hippocampal neurogenesis (Schouten et al., 2012). Additionally, a number of microRNAs such as miR-9, miR-let7b and miR-378 have been shown to suppress TLX expression resulting in decreased NSC proliferation and accelerated neuronal differentiation (Huang et al., 2015; Zhao et al., 2010; Zhao et al., 2009). In summary, TLX maintains NSCs in their proliferative state through a variety of autonomous and/or parallel pathways controlled by different genes, and disruption of these genes results in altered adult neurogenesis and brain plasticity (Islam & Zhang, 2015).

Mice with spontaneous deletion of TLX present with impaired neurogenesis, synaptic plasticity, dendritic complexity and hippocampal-dependent behaviours during adulthood (Christie et al., 2006; Kozareva et al., 2017a; O'Leary et al., 2016a; Young et al., 2002). Interestingly, we have recently shown that a lack of TLX expression in the hippocampus resulted in microglial activation (Kozareva et al., 2017a). Our group has also previously demonstrated using both *in vitro* and *in vivo* approaches that an inverse relationship exists between levels of TLX and the microglial derived pro-inflammatory cytokine IL-1β in the hippocampus (Green and Nolan, 2012b; Kozareva et al., 2017a). Specifically, we found a reduction in TLX expression on hippocampal NPCs *in vitro* after administration of IL-1β (Green and Nolan, 2012b), a dramatic increase of IL-1β in the hippocampi of TLX knockout mice (Kozareva et al., 2017a), and a protective capacity of TLX to mitigate the negative effects of IL-1β on NPCs

(O'Leime et al., 2018). Given that CX3CR1^{KO} mice present with increased microglial activation and hippocampal IL-1β (Rogers et al., 2011) it is possible that in the absence of TLX, intracellular communication between microglia and NPCs through CX3CL1/CX3CR1 is impaired. Thus, the aim of the present study was to determine whether a disturbance in CX3CL1/CX3CR1 communication mediates an impairment in TLX-related pathways (upstream regulators and downstream targets of TLX) which may have subsequent effects on hippocampal neurogenesis.

3.2 Materials and Methods

3.2.1 Animals

Two-month-old male homozygous CX3CR1-GFP mice with CX3CR1 deficiency (CX3CR1^{KO} on C57BL/6 genetic background, n=16) and wild type (n=16) controls were group housed under standard housing conditions (temperature 21°C and relative humidity 55%), with food and water available *ad libitum*. The GFP gene was knocked-in under the CX3CR1 promoter (Jung et al., 2000). The mice were obtained from the Jackson Laboratory (B6.129P-CX3CR1^{tm1Litt}/J; mouse strain datasheet #005582) and were generation N13F2 (backcrossed for 13 generations on C57BL/6 and second filial generation was used). In order to confirm the knockout in the animals used, we employed PCR and used the Jackson Laboratory Protocol (Stock number: 005582) called Cx3cr1^{tm1Litt}alternate1 (see https://www2.jax.org/protocolsdb/f?p=116:5:0::NO:5:P5_MASTER_PROTOCOL_ID,P5_JRS_CODE:27927,005582). Two-month-old male Nr2e1$^{-/-}$ (TLX knockout) knockout mice and wildtype controls (129S1/SvImJ background) were housed under

standard housing conditions (temperature 21°C and relative humidity 55%), with food and water available *ad libitum*. Breeding pairs were kindly provided by Prof. Elizabeth Simpson, University of British Colombia. Nr2e1$^{-/-}$ mice exhibit a spontaneous deletion of the entire TLX allele, including all nine exons. However, the deletion of TLX does not affect the transcription of neighbouring genes (Kumar et al., 2004). All experiments were conducted in accordance with the European Directive 2010/63/EU, and under an authorization issued by the Health Products Regulatory Authority Ireland and approved by the Animal Ethics Committee of University College Cork.

3.2.2 BrdU administration and tissue preparation

Bromodeoxyuridine (BrdU; Sigma) was administered (4 x intraperitoneal injections over the course of 6 hours at 75µg/10mL/kg) to the Nr2e1$^{-/-}$ and wildtype mice at postnatal day 42. At postnatal day 56 these mice were euthanized with an intraperitoneal injection of anaesthetic (0.1mL/kg) and transcardially perfused using a 0.9% phosphate buffered saline solution followed by 4.0% paraformaldehyde in phosphate buffered saline. After overnight invubation in 4% paraformaldehyde, brains were incubated in 30% sucrose until they sank, and subsequently flash frozen using liquid nitrogen. Coronal sections (40µm) through the hippocampus were collected directly onto slides in a 1:6 series, then stored at -80ºC.

3.2.3 Immunohistochemistry

To determine the survival of adult-born hippocampal neurons in Nr2e1$^{-/-}$ mice, sections were double-labelled with BrdU and the neuronal marker NeuN. DNA was denatured in sections by incubation in 2M HCl for 45 min at 37 ºC, renatured in 0.1M

sodium tetraborate (pH 8.5) and then blocked in 3% normal donkey serum (NDS; Sigma D9663). Slides were incubated with anti-BrdU antibody (Abcam; AB6326; 1:250) then incubated with AlexaFluor594 donkey anti-rat (Abcam; AB150156; 1:500) and with NeuN (Millipore; MAB377; 1:100). Finally sections were incubated with AlexaFluor488 donkey anti-mouse (Abcam; AB150105; 1:500), washed and coverslipped using anti-fade medium (DAKO; S3023). The number of microglia in the hippocampi of these animals was assessed by staining for ionized calcium binding adaptor molecule 1 (Iba-1). Sections were washed, incubated in 3% NDS and then in anti-Iba-1 (Wako; 019-19741; 1:1000) overnight. Sections were then incubated in AlexaFluor488 donkey anti-rabbit and were subsequently counterstained with DAPI (Sigma; D9642; 1:5000) and coverslipped with anti-fade mounting medium (DAKO; S3023).

3.2.4 Image analysis and cell quantification

Images were obtained using an Olympus FV1000 scanning laser confocal system (Biosciences Imaging Centre, Department of Anatomy and Neuroscience, University College Cork, Ireland). Z-stack images with a 1.10 μm or 4.4 μm step size were collected using a 10X objective (BrdU/NeuN) or 20X objective (Iba1/DAPI), respectively. The DG was imaged bilaterally on all sections. Cell quantification and area measurements were performed using the image processing software ImageJ (National Institute of Health; USA; Schneider et al., 2012). Systematic random sampling was employed for all cell quantifications and for area quantification the area of interest (i.e. the DG or the microglia soma) was outlined manually and its area calculated using ImageJ.

3.2.5 Total RNA extraction and cDNA synthesis

Animals were sacrificed by cervical dislocation and the hippocampus was dissected out and stored in solution that stabilizes and protects cellular RNA (RNA*later*; Sigma) for 48 hours at 4ºC, after which the RNAlater was removed and the tissue was frozen at -80°C until subsequent use. Samples were processed according to the GenElute kit protocol (Sigma; RTN350). Total RNA yield and purity were determined using the Nanodrop System (Thermo Scientific). Synthesis of complementary DNA (cDNA) was performed using 0.5 μg of normalized total RNA from each sample using ReadyScript cDNA synthesis mix (Sigma; RDRT-25RXN).

3.2.6 microRNA extraction and cDNA synthesis

Total mRNA was isolated from hippocampal samples using mirVANA miRNA Isolation Kit (Life Technologies) according to manufacturer's instructions. Total RNA yield and quality were verified using the Nanodrop2000 spectrophotometer (ThermoScientific, Waltham, MA, USA). RNA was reverse-transcribed to cDNA using hairpin primers specific to each microRNA gene of interest on Applied Biosystem's GeneAmp PCR System 9700.

3.2.7 Quantitative RT-PCR analysis and microRNA quantification

Quantitative real-time PCR (qRT-PCR) was performed in duplicate and triplicate in a 96-well plate (Applied Biosystems) and captured in real time using the StepOne Plus System (Applied Biosystems). Gene expression levels were calculated as the average ct value of three replicates for each sample relative to the expression of the

housekeeper gene *Tfrc* and quantified using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Primer sequences were: 5`-CCCAAGTATTCTCAGATATGATTTCAA-3' (forward) and 5'-AAAGGTATCCCTCCAACCACTC-3' (reverse) for *Tfrc*; 5'-CTGGGCCCTGCAGATACTC-3' (forward) and 5'-GGTGGCATGATGGGTAACTC-3' (reverse) for *Nr2e1* (TLX); 5'-AGCCCGCTTCTGCAGGA-3' (forward) and 5'-AAAGGCTCAGAGAAGCTGCG-3' (reverse) for *Bmp4*; 5′-CAGGGTTTTCTCTTGCAGAAG A-3′ (forward) and 5′-ATGTCCAATCCTGGTGATGTCCG-3′ (reverse) for *p21*; 5′-GTGGTCTGCCAGCTAAAGGTGA-3′ (forward) and 5′-TCAGACTTTTGTAATTTGTGAATGCT-3′ (reverse) for *Pten*; 5'-TTAACGCAAAAACCGTGATG-3' (forward) and 5'-GAAGCGCCTAACGTACCACT-3' (reverse) for *Sox2* and 5′- GTC TTC ACG TTC GGT CTG GT-3′ (forward) for *Cx3cr1*.

qRT-PCR was performed on the small RNA-enriched samples using probes (6 carboxy fluorescein-FAM) designed by Applied Biosystems (Carlsbad, CA, USA): miR-let7b, miR-9, miR-378. qRT-PCR was carried out on the StepOnePlus PCR machine (Applied Biosystems). Samples were heated to 95°C for 10 min, and then subjected to 40 cycles of amplification by melting at 95°C and annealing at 60°C for 1 min. Experimental samples were run in technical triplicates with 1.33 μL complementary DNA per reaction. To check for amplicon contamination, each run also contained template free controls for each probe used. The non-coding small nuclear RNA component U6, which is highly conserved and expressed across species, was used as

the endogenous control. U6 was stably expressed in all samples and differences in miRNA expression were presented as fold change from control.

3.2.8 Statistical analysis

All data were analysed using SPSS statistical software (SPSS 17.0, Chicago, Il). Data were analysed by an independent-sample t-test and an alpha level of 0.05 was used as criterion for statistical significance. All data are presented as mean ± SEM.

3.3 Results

3.3.1 Negative correlation between neurogenesis and microglial activation in the hippocampus as a result of TLX deficiency

There was a significant negative correlation between the mean number of BrdU/NeuN-positive cells and the mean number of Iba1-positive cells in the dentate gyrus in mice with a spontaneous deletion of TLX ($Nr2e1^{-/-}$; r = 0.871, n=8, p = 0.004, Figure 3.1). We found the same negative correlation between cell soma size of microglia and number of BrdU/NeuN-positive cells in the absence of TLX ($Nr2e1^{-/-}$; r = 0.735, n=8, p = 0.037, Figure 3.1). Representative images from wildtype controls and mice with spontaneous deletion for TLX show reduced number of BrdU/NeuN-positive cells as well as atrophied DG morphology (Figure 3.1) and increased number of microgia in the DG of $Nr2e1^{-/-}$ mice (Figure 3.1).

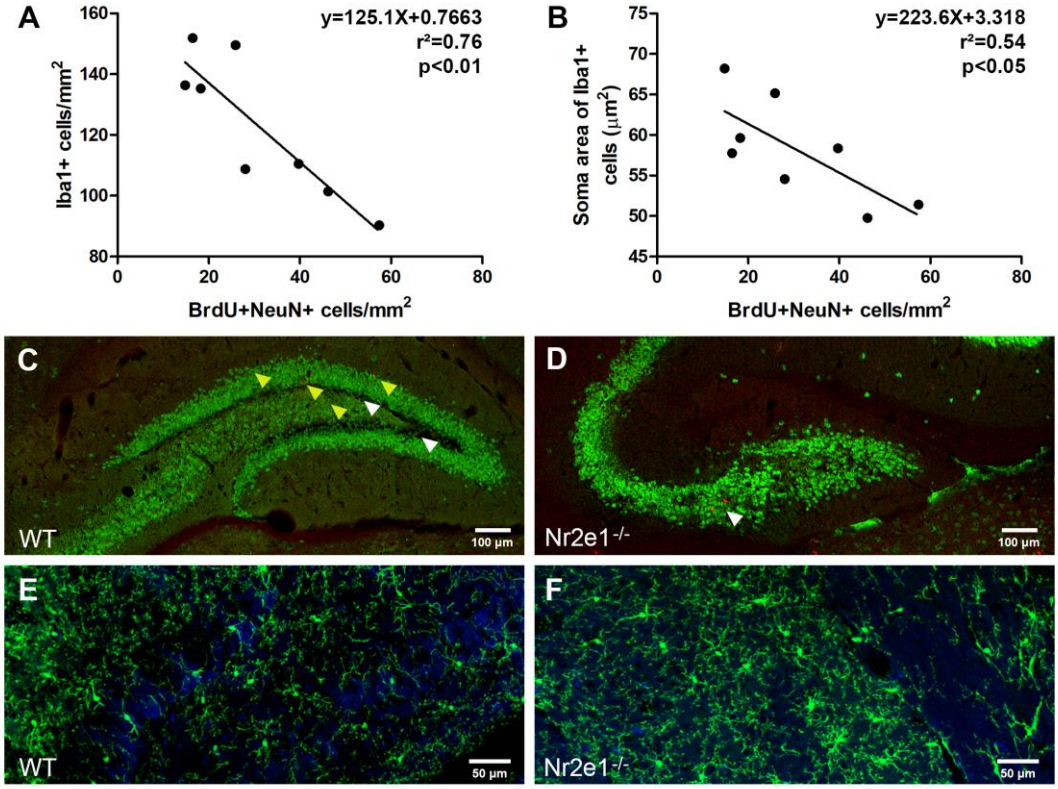

Figure 3.1: Negative correlation between neurogenesis and microglia activation in the hippocampi of TLX deficient mice. Correlation between number of Iba1+ cells and the number of BrdU+NeuN+ cells per mm^2 **(A)** and correlation between the soma area (μm^2) of Iba1+ cells and the number of BrdU+NeuN+ cells **(B)** in the DG of TLX knockout ($Nr2e1^{-/-}$) mice. Data graphed as means (n=8). Representative confocal images from wildtype (WT; **C**) and TLX knockout ($Nr2e1^{-/-}$; **D**) of coronal sections through the DG immunohistochemically stained with BrdU (red; white arrow heads), NeuN (green) and BrdUNeuN (orange, yellow arrowheads) at 10X magnification. Scale bar = 100 µm. Representative confocal images from wildtype (WT; **E**) and TLX knockout ($Nr2e1^{-/-}$; **F**) of coronal sections through the DG immunohistochemically stained with Iba1 (green) and the nuclear stain DAPI (blue) at 20X magnification. Scale bar = 50 µm.

Additionally, qRT-PCR of the hippocampi of $CX3CR1^{KO}$ and wildtype control mice confirmed the absence of the gene in the formed and its presence in the latter (Figure 3.2).

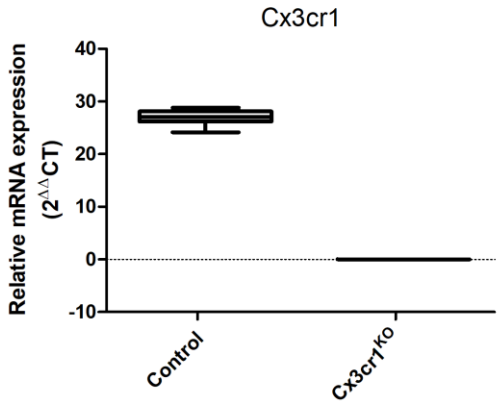

Figure 3.2: mRNA expression of CX3CR1 is detected in wildtype controls but not CX3CR1KO mice. Relative mRNA expression of Cx3cr1 in the hippocampus of wildtype controls and $CX3CR1^{KO}$ mice. Box and whisker plots show mean, first and third quartiles, and maximum and minimum values (n=8 per group).

3.3.2 mRNA expression of TLX but not its transcription activator Sox2 is downregulated in the hippocampus in the absence of CX3CR1

TLX gene expression was significantly decreased in the hippocampus of $CX3CR1^{KO}$ mice compared to controls (t (14) = 2.115, p = 0.05; Figure 3.3). There was no difference in expression of the TLX regulator Sox2 between wildtype controls and $CX3CR1^{KO}$ mice (Figure 3.3).

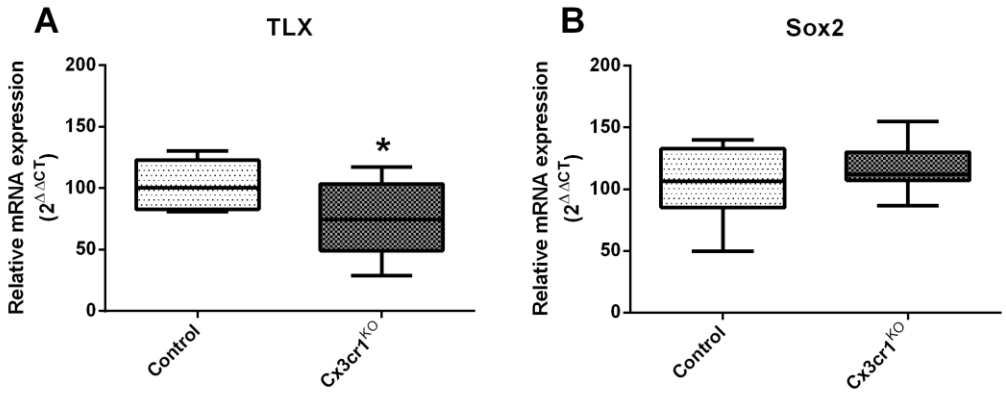

Figure 3.3: mRNA expression of TLX but not its transcription activator Sox2 is downregulated in the hippocampus in the absence of CX3CR1.
Relative mRNA expression of TLX (A) and Sox2 (B) in the hippocampus of wildtype control and CX3CR1^{KO} mice. All values were adjusted to the relative expression of the housekeeping gene *Tfrc*. Box and whisker plots show mean, first and third quartiles, and maximum and minimum values (n=8 per group). *$p \leq 0.05$, independent-sample t-test.

3.3.3 Lack of CX3CR1 is not associated with changes in expression of microRNAs regulating TLX in the hippocampus

Upon examination of upstream microRNAs regulating TLX we found no change in expression of miR-let7b (Figure 3.4) or miR-9 (Figure 3.4) in hippocampal tissue between control and CX3CR1^{KO} mice. Nonetheless, we observed a trend towards increased expression of the TLX suppressor miR-378 in CX3CR1^{KO} mice compared to the wildtype controls (t (14) = 1.925, p = 0.07; Figure 3.4).

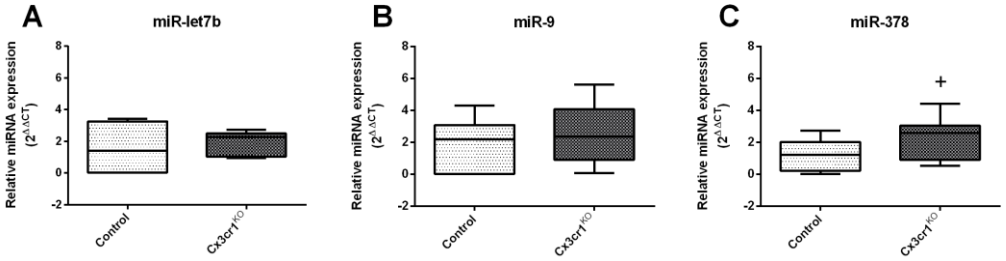

Figure 3.4: Lack of CX3CR1 is not associated with changes in expression of microRNAs regulating TLX in the hippocampus.

microRNA expression in wildtype controls and CX3CR1^{KO} mice for miRNAs suppressing TLX expression: miR-let7b (A), miR-9 (B) and miR-378 (C). Values are expressed relative to the non-coding RNA component U6. Box and whisker plots show mean, first and third quartiles, and maximum and minimum values (n=8 per group). +p = 0.07, independent-sample t-test.

3.3.4 TLX target genes BMP4 and pten, but not p21 are upregulated in the hippocampus of CX3CR1^{KO} mice

When genes targeted by TLX were measured, we detected significantly higher relative expression of Bmp4 (t (14) = 2.228, p = 0.04; Figure 3.5) and Pten (t (14) = 2.718, p = 0.02; Figure 3.5) in the hippocampi of CX3CR1^{KO} mice compared to wildtype controls. There was no alteration in relative expression of the cyclin dependent kinase inhibitor gene p21 another target of TLX, between the hippocampi of wildtype controls and the CX3CR1^{KO} mice (Figure 3.5**)**.

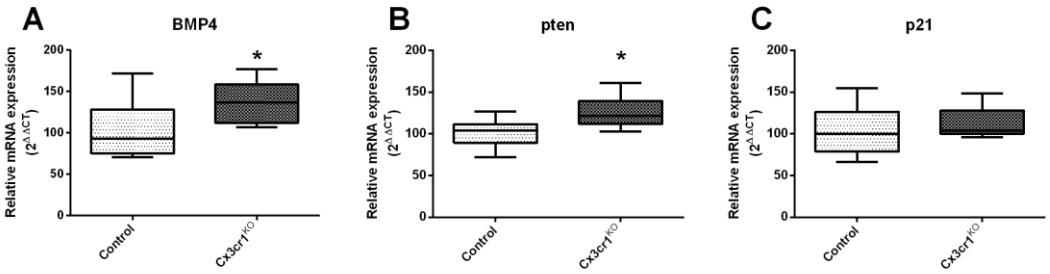

Figure 3.5: TLX target genes BMP4 and pten, but not p21 are upregulated in the hippocampus of CX3CR1KO mice.
Relative mRNA expression of BMP4 (A), pten (B) and p21 (C) in the hippocampus of wildtype control and CX3CR1^{KO} mice. All values were adjusted to the relative expression of the housekeeping gene *Tfrc*. Box and whisker plots show mean, first and third quartiles, and maximum and minimum values (n=8 per group). *p < 0.05, independent-sample t-test.

3.4 Discussion

Here we have shown that in the absence of CX3CR1, TLX transcription within the hippocampus is reduced. This reduction in TLX relative mRNA abundance is not associated with changes in miR-let7b, miR-9 or miR-378, the upstream repressors of

the gene, though there was a trend towards increased expression of the TLX repressor miR-378. Furthermore, no change was observed in the expression of the TLX promoter and activator Sox2. However, CX3CR1^{KO} mice exhibited an increase in Bmp4 expression, a downstream target of TLX, involved in gliogenesis and astrocyte differentiation. In addition, in the absence of CX3CR1, hippocampal expression of the TLX repressor target Pten but not p21 was increased. Collectively, these data suggest that absence of CX3CR1 promotes downregulation of TLX expression and its downstream targets, without influencing the regulators of the gene. Thus TLX may be an important target in the crosstalk between microglia and hippocampal NSCs.

We hypothesize that the CX3CR1 signalling pathway in microglia and TLX signalling pathways in NPCs interact to maintain homeostasis in the neurogenic niche in the adult hippocampus, which is supported by our observation of a decrease of TLX transcription in CX3CR1^{KO} mice. This is also in line with the emerging evidence that microglia regulate neurogenesis in temporal- and spatial-dependent manner and that microglia are proposed as a key mediator and integrator of information that may influence the neurogenic niches (reviewed by Sato, 2015). Here we show a significant negative correlation between hippocampal neurogenesis and microglia number and soma size in the absence of TLX which supports our previous demonstration that the absence of TLX results in an activated microglial phenotype, increased levels of endogenous IL-1β and impaired hippocampal neurogenesis (Kozareva et al., 2017) and implicate TLX as a mediator of NS/PC-microglial communication. However, the temporal relation between the associations we observed remains unclear. Further

studies examining whether granule neurons and NPCs in the hippocampi of TLX deficient mice have impaired secretion of CX3CL1 would provide valuable information whether the activated microglia in TLX knockout mice has been promoted through a CX3CL1/CX3CR1-mediated pathway. Additionally, isolating microglia from the hippocampi of TLX deficient mice and examining how these microglia behave in response to pro- and anti-inflammatory stimulation with cytokines would provide insight into the temporal relationship of our findings. Since CX3CR1 deficient mice have been shown to present impaired hippocampal-dependent but not olfactory-dependent memory performance (Rogers et al., 2011; Reshef et al., 2014) it would also be of interest to compare the two neurogenic niches (the SGZ and the subventricular zone) directly in terms of the expression of TLX and other neurogenic markers in the context of lack of CX3CR1 expression. This would thus illustrate whether TLX mediates NPC-microglia crosstalk in a region-specific manner.

While it has been demonstrated that microglia have a direct effect on NSCs, the mechanisms by which microglia exercise their influence on NSCs remain largely unknown (Gemma and Bachstetter, 2013; Sato, 2015; Lira-Diaz and Gonzalez-Perez, 2016). Our findings suggest that deletion of CX3CR1 expression on microglia facilitate the activation of down- but not up- stream pathways of TLX. As there was no upregulation in mRNA expression of the transcription factor Sox2 in the hippocampi of $CX3CR1^{KO}$ mice, it is possible that the reduction of TLX gene transcription observed is regulated through a self-repression mechanism. This is supported by the fact that the transcriptional regulation of NPC by Sox2 and TLX is

autonomous such that both can act independently for the potentiation of cell proliferation and the repression of cell differentiation in order to maintain the undifferentiated and self-renewable state of progenitors within the neurogenic niche (Pevny and Nicolis, 2010; Shi et al., 2008).

A number of miRNAs, such as miR-9, miR-137, miR-let7d, and miR-let7b, have been shown to regulate neurogenesis rate and progression through suppression of TLX *in vitro* (Hu et al., 2014; Huang et al., 2015; Ni et al., 2014; Sun et al., 2011a; Zhao et al., 2009; Zhao et al., 2013a) and to be associated with suppression of TLX expression *in vivo* in hippocampi of adult mice (Murai et al., 2014). Thus, we examined whether the reduction of TLX gene transcription in adult CX3CR1^{KO} mice was associated with upregulation of miR-let7b and/or miR-9. Our results showed no change in the expression of either miR-let7b or miR-9 in the absence of CX3CR1 in the hippocampi of knockout and wildtype mice. It has been shown that miR-let7b and miR-9 are heterochronic switch genes, which induce acceleration in NSC differentiation and reduction in their proliferation by targeting TLX expression (Zhao et al., 2010; Zhao et al., 2009). Interestingly, both miRNAs not only bind to TLX, but their expression is inversely related to that of TLX such that silencing and overexpression of the miRNAs causes an inverse increase or reduction, respectively, in TLX (Zhao et al., 2010; Zhao et al., 2009). This phenomenon has been demonstrated in adult hippocampal NPC cultures and in the embryonic developing brain for both miR-9 and miR-let7b (Zhao et al., 2010; Zhao et al., 2009) as well as in retinal progenitor cells for miR-let7b (Ni et al., 2014). As these processes may occur autonomously of

increased NPC neuronal differentiation (which would be induced by miR-let7b and/or miR-9) combined with the fact we did not observe any change in miR-let7b and/or miR-9 expression, it follows that the reduction in TLX in CX3CR1^{KO} mice is independent of either the TLX-miR-let7b regulatory loop or the TLX-miR-9 feedback pathway and is brought about through a different signalling route. This further supports our conjecture that the downregulation of TLX observed in the absence of CX3CR1 could be a result of a self-repression mechanism, thereby positioning TLX as a potential target or co-regulator of the CX3CR1/CX3CL1 pathway.

Mir-378 has been shown as important enhancer of cell survival through reduction of apoptosis (Krist et al., 2015; Lee et al., 2007) as well as promoter of NSC reduced proliferation and enhanced differentiation (Huang et al., 2015). We observed a trend towards increased expression of miR-378 in the hippocampus of CX3CR1^{KO} mice compared to controls. Similar to miR-let7b and miR-9, miR-378 has been shown to exert its effect on NSCs through binding and suppressing the expression of TLX (Huang et al., 2015) coupled with an upregulation of two of TLX' downstream targets – p21 and PTEN (Huang et al., 2015). Given the trend towards increased miR-378 expression, we examined the mRNA levels of p21 and pten and observed an increase of pten but no change in p21 expression in the hippocampi of CX3CR1^{KO} mice. However, a limitation of our study is the fact that we examined expression levels in the whole hippocampus, rather than specifically in the DG where neural stem cells predominantly reside. The mechanisms by which TLX suppresses both of those genes have been extensively studied: the repression of both genes occurs through an

interaction between TLX and the histone demethylase LSD1 (Gui et al., 2011; Sun et al., 2010). In the case of pten, however, the histone deacetylases (HDAC) 1 and 2 are recruited to form complex with the REST corepressor 1 (CoREST), which results in demethylation of H3K4 at the proximal region of pten and hence its suppression ((Yokoyama et al., 2008); See Figure 3.6) p21 repression on the other hand, can result from the interaction between TLX and LSD1 with HDAC5 (Sun et al., 2007) or from p53-TLX – dependent signalling ((Niu et al., 2011); See Figure 3.6). Within NSCs, it has been shown that blocking TLX-mediated suppression of both pten and p21 resulted in a reduction of NSC proliferation (Sun et al., 2007) and an increase in quiescent hippocampal NSCs rather than an increase in differentiating NSCs, which was coupled with activation of pten and p21 signalling pathways (Niu et al., 2011). Thus, in the context of the present study, in the absence of CX3CR1 on microglia TLX expression in NPCs is reduced, which is associated with a trend towards increased expression of the TLX suppressor miR-378 and an increase in the TLX downstream target pten but not p21. Hence, it is possible that the reduction in hippocampal neurogenesis observed in $CX3CR1^{KO}$ mice by others (Reshef et al., 2014; Rogers et al., 2011) results from activation of TLX-suppressing signalling pathways that inhibit activation of quiescent NSCs and maintain them in their non-proliferative state through pten signalling mechanisms. Interestingly, it has been shown that attenuation of pten increases p21 stability in cancer stem cells (Lin et al., 2007), which may explain why we observed an increase in pten only but not in p21.

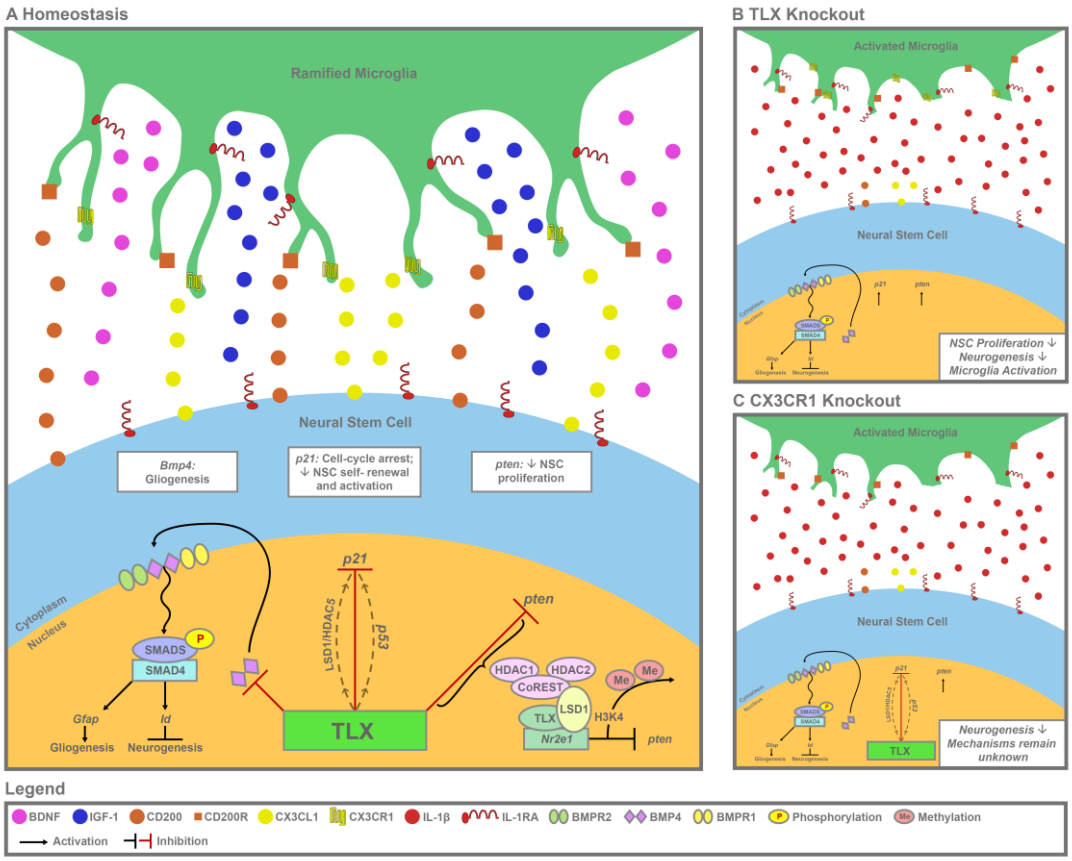

Figure 3.6: Schematic diagram illustrating possible mechanisms by which NSC and microglia interact. Under homeostasis (A) TLX acts as a transcriptional repressor of variety of genes in order to maintain NSC in their proliferative and non-differentiative state by inhibiting gliogenesis (Bmp4 pathway), cell-cycle arrest (p21 pathway) and decreased proliferation (pten pathway). Microglia send neurotrophic signals to NSCs such as BDNF and IGF-1 and receive output from NSCs through CX3CL1/CX3CR1 and CD200/CD200R mediated pathways. In the TLX knockout mouse model (B), microglia become activated (retracted processes) and NSC proliferation and neurogenesis has been significantly decreased, coupled with increased levels of endogenous IL-1β. In the CX3CR1 knockout mouse model (C) microglia activation and reduction in neurogenesis are coupled again with increased levels of endogenous IL-1β. We have also observed that TLX expression is reduced, while Bmp4 and pten are upregulated. The mechanisms regulating these processes remain unknown.
GFAP: Glial fibrillary acidic protein; Id: Inhibitor of DNA-binding/differentiation protein; BMP4: Bone morphogenic protein 4; BMPR1: Bone morphogenic protein receptor, type 1; BMPR2: Bone morphogenic protein receptor, type 2; SMADS: homologies to the Caenorhabditis elegans SMA ("small" worm phenotype) and Drosophila MAD ("Mothers Against Decapentaplegic") family of genes; P: phosphorylation; SMAD4: Mothers against decapentaplegic homolog 4; LSD1: Lysine-specific histone demethylase 1A; HDAC5: histone deacetylase 5; p53: tumor protein TP53; p21: cyclin-dependent kinase inhibitor 1; NSC: neural stem cell; pten: phosphatase and tensin homolog; HDAC1: Histone deacetylase 1; HDAC2: Histone deacetylase 2; CoREST: REST (RE1-silencing transcription factor) corepressor 1; TLX: Protein Tailleess Homolog; Nr2e1: Nuclear receptor subfamily 2 Group E Member 1; H3K4: trimethylation of histone H3 at lysine 4; Me: methylation.

TLX is also involved in the transcriptional repression of the BMP4-SMAD signalling pathway which activates astrogenesis ((Qin et al., 2014); See Figure 3.6). Specifically, TLX prevents the binding of BMP ligands to their type I (BMPR1; Figure 3.6) and

type II (BMPR2; Figure 3.6) receptors; blocking the activation of these receptors prevents the phosphorylation of regulatory SMADs and their dimerization with the common cofactor SMAD4 (Figure 3.6; (Qin et al., 2014)). Under normal physiological conditions, the SMAD complex transcriptionally activates downstream targets which promote astrogenesis (GFAP) and inhibits neurogenesis via suppressing inhibitors of differentiation (*Ids*; Figure 3.6; (Qin et al., 2014). Interestingly, in the present study $CX3CR1^{KO}$ mice displayed an increase in relative mRNA expression of BMP4, the downstream target of TLX, responsible for astrogenesis. Thus, it would be of interest to examine in future studies whether the reduction of hippocampal neurogenesis in $CX3CR1^{KO}$ mice is coupled with an increase in hippocampal astrogenesis. This is of particular importance, given that we previously observed an increased level of endogenous IL-1β coupled with increased microglia activation in TLX knockout mice (Kozareva et al., 2017). Similarly. CX3CR1 deficient mice present with increased microglia activation and increased endogenous hippocampal IL-1β (Rogers et al., 2011; Reshef et al., 2014). Due to the fact that astrocytes are major producers of IL-1β in the central nervous system (Moynagh, 2005; Srinivasan et al., 2004), they may act as the "middle man" in the cascade leading to impaired neurogenesis in the context of CX3CR1 and/or TLX deficiency. Thus studies investigating the cause of inflammation in the absence of TLX will be key to determining the relationship between TLX and CX3CR1/CX3CL1 signalling.

It has previously been shown that microglia development and adult hippocampal neurogenesis are impaired in $CX3CR1^{KO}$ mice (Pagani et al., 2015; Rogers et al.,

2011), but has more recently been suggested that the decrease of neurogenesis observed in the CX3CR1 deficient mice is due to pathways independent of CX3CL1 (Sellner et al., 2016). Thus understanding the precise interactions and signalling mechanisms between and within microglia and NPCs may aid our understanding of diseases associated with defective microglia-neuronal crosstalk as well as with the neuropathology of ageing (Jurgens and Johnson, 2012). Here we show that CX3CR1 deficiency in the hippocampus leads to the activation of TLX-dependent pathways within NSCs that may inhibit their self-renewal and promote their adoption of an astrocytic fate. We propose that TLX is a mediator in maintaining homeostasis between microglia and NPCs. However, future studies are needed to examine whether TLX repression leads to impaired proliferation and neurogenesis and /or gliogenesis through CX3CL1/CX3CR1-dependent mechanisms.

Chapter 4

Deletion of Tlx and social isolation impairs exercise-induced neurogenesis in the adolescent hippocampus

Danka A. Kozareva, Olivia F. O'Leary, John F. Cryan and Yvonne M. Nolan*

Department of Anatomy and Neuroscience, University College Cork, Ireland

APC Microbiome Institute, University College Cork, Ireland

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Abstract

Adolescence is a sensitive period of neurodevelopment during which life experiences and the surrounding environment can have profound effects on the brain. Neurogenesis is a neurodevelopmental process of generating functional neurons from neural stem cells. Hippocampal neurogenesis occurs throughout the lifespan and has been shown to play a role in learning, memory and in mood regulation. In adulthood it is influenced by extrinsic environmental factors such as exercise and stress. Intrinsic factors that regulate hippocampal neurogenesis include the orphan nuclear receptor TLX (Nr2e1) which is primarily expressed in the neurogenic niches of the brain. While mechanisms regulating adult hippocampal neurogenesis have been widely studied, less is known on how hippocampal neurogenesis is affected during adolescence. Thus, the aim of this study was to investigate the influence of both TLX and isolation stress on exercise-induced increases in neurogenesis in running and sedentary conditions during adolescence. Single- (i.e. isolation stress) wild type and $Nr2e1^{-/-}$ or pair-housed wild type mice were housed in sedentary conditions or allowed free access to running wheels for 3 weeks during the adolescent period. A reduction of neuronal survival was evident in mice lacking TLX, and exercise did not increase hippocampal neurogenesis in these $Nr2e1^{-/-}$ mice. This suggests that TLX is necessary for the pro-neurogenic effects of exercise during adolescence. Interestingly, although social isolation during adolescence did not affect hippocampal neurogenesis, it prevented an exercise-induced increase in neurogenesis in the ventral hippocampus. Together these data demonstrate the importance of intrinsic and extrinsic factors in promoting an exercise-induced increase in neurogenesis at this key point in life. *Key words:* adult neurogenesis, adolescence, stress, exercise, TLX

4.1 Introduction

Adolescence is a critical phase of development associated with plasticity-driven organization of neural circuits in the hippocampus, prefrontal cortex and amygdala (Pattwell et al., 2011; Selemon, 2013). It is also a key period for susceptibility to stress and the emergence of neurobiological disorders such as schizophrenia, depression and anxiety (Green and Nolan, 2014; Hueston et al., 2017; O'Connor and Cryan, 2014; Paus et al., 2008). By approximately postnatal day (P) 30 in rodents, dentate gyrus (DG) formation, cerebellar neurogenesis and myelogenesis are completed, and neurogenesis (the birth of new neurons) is restricted to the niches of the brain where the process persists through adulthood – the subgranular zone (SGZ) of the DG of the hippocampus and the subventricular zone (Lemasson et al., 2005; Li et al., 2009).

Hippocampal neurogenesis has been widely studied in the adult brain and is known to be regulated by several extrinsic and intrinsic factors (Aimone et al., 2014; Gregoire et al., 2014). For example, extrinsic factors such as stress and exercise have been shown to decrease or increase adult hippocampal neurogenesis, respectively (Levone et al., 2015; van Praag et al., 1999a). However, comparatively fewer studies have investigated the impact of these extrinsic factors on hippocampal neurogenesis in the adolescent brain (Abel and Rissman, 2013; Kirshenbaum et al., 2014; Wei et al., 2011). In adult rodents, exercise has been shown to enhance learning and memory (Creer et al., 2010; Marlatt et al., 2012; van Praag et al., 1999a), protect against stress-induced depression and anxiety-like behaviours (Duman et al., 2008; Grippo et al., 2014) and protect against cognitive deficits in neurodegenerative disorders (Barbour

et al., 2007; Cotman et al., 2007; Ryan and Nolan, 2016). How exercise can facilitate processes as diverse as spatial learning and memory, anxiety and responses to stress is not yet clear. However, accumulating evidence suggests that the hippocampus is functionally segregated along its dorsoventral axis in rodents such that the dorsal hippocampus (dHi) plays a predominant role in spatial learning and memory while the ventral hippocampus (vHi) plays a predominant role in anxiety and the stress response (Bannerman et al., 2004; Fanselow and Dong, 2010). Similarly, there is an emerging view that neurogenesis might also be similarly functionally segregated along this axis (O'Leary and Cryan, 2014; Tanti and Belzung, 2013). Intrinsic factors that control hippocampal neurogenesis include the orphan nuclear receptor TLX (Nr2e1) which is primarily expressed in the neurogenic niches of the postnatal brain and has been implicated as an important regulator of neural stem cells by maintaining them in their proliferative and non-differentiated state (Roy et al., 2004; Shi et al., 2004). In a spontaneous deletion mouse model, adult TLX knock out ($Nr2e1^{-/-}$) mice display altered neurogenesis and synaptic plasticity, as well as an impairment of dendritic structures in the DG. These mice also present with an aggressive phenotype and display cognitive impairments in hippocampal-dependent tasks (Christie et al., 2006; Young et al., 2002). Interestingly, some of these effects are apparent in adolescence (O'Leary et al., 2016a; O'Leary et al., 2016b).

Taken together, while mechanisms regulating adult hippocampal neurogenesis have been thoroughly investigated, less is known about how neurogenesis is affected during the adolescent period. Moreover, the impact of facilitators and impeders of

neurogenesis, such as exercise and stress, on the adolescent brain has yet to be established. This is surprising given that adolescence is a critical period for the maturation of neurons as well as a time during which profound social and physiological change occurs. Thus, the aim of this study was to investigate (1) the impact of exercise during adolescence on hippocampal neurogenesis; (2) the role of the intrinsic factor TLX on exercise-induced changes in hippocampal neurogenesis and (3) whether social isolation stress influences exercise-induced changes in hippocampal neurogenesis. Given the growing evidence of segregated effects on neurogenesis along the dorsoventral axis of the hippocampus, we examined the impact of exercise, TLX, and social isolation on neurogenesis in the dorsal versus ventral hippocampus.

4.2 Materials and Methods

This animal study was conducted in strict compliance with the European Directive 2010/63/EU, and under an authorization issued by the Health Products Regulatory Authority Ireland and approved by the Animal Ethics Committee of University College Cork (UCC). Breeding pairs of $Nr2e1^{-/-}$ mice exhibiting a spontaneous deletion of TLX were kindly provided by Prof. Elizabeth Simpson (University of British Colombia) and were generated as previously described (Wong et al., 2010). Male $Nr2e1^{-/-}$ mice and wild type (WT) littermate controls on a BL6129 background were singly-housed upon weaning and given *ad libitum* access to food and water under a 12-12 h light/dark cycle. At 4 weeks of age (P28), mice received 4 x bromodeoxyuridine (BrdU; 75mg/kg, i.p., Sigma Cat# B5002) injections at 2-hour intervals to label newly-born cells. Half of the animals from each genotype were then

housed with free access to a running wheel for 3 weeks (Med Associates Inc Cat# ENV-044), thus there were 4 experimental groups (WT sedentary, WT running, Nr2e1$^{-/-}$ sedentary; Nr2e1$^{-/-}$ running; n = 5-6 per group; Figure 4.1). Male mice were collected from 11 litters in total. For welfare reasons Nr2e1$^{-/-}$ mice have to be singly housed due to their aggressive phenotype (Young et al., 2002). Thus, their corresponding wild type littermates were also singly housed. Since single housing is a social isolation stressor, we sought to examine whether this chronic stress influenced the effect of exercise on hippocampal neurogenesis in WT mice (Figure 4.1).

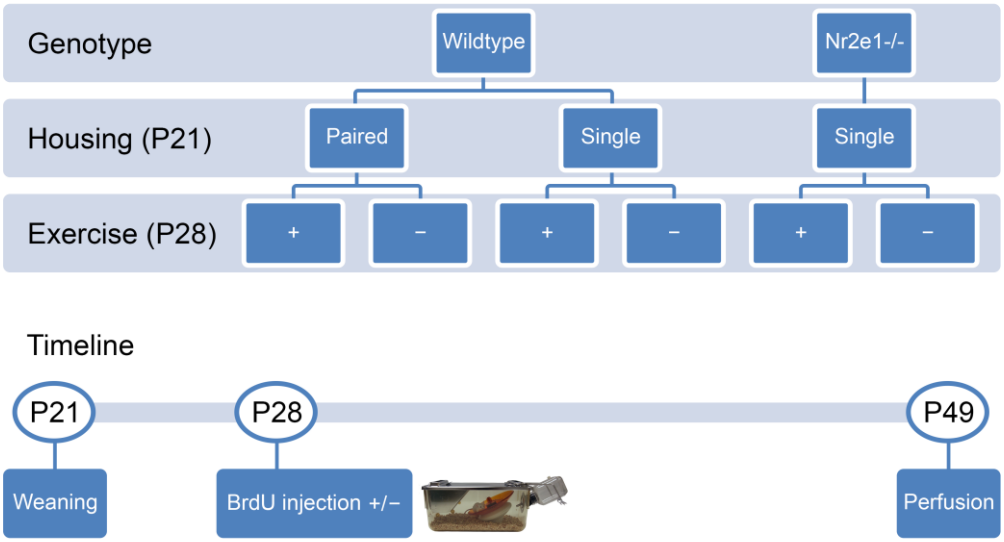

Figure 4.1: Experimental design.
Outline of the experimental groups and timeline illustrating the duration of the experiment.

Three weeks following BrdU administration and initiation of exercise, mice (P49) were anesthetized with Euthasol (1.0 mL/kg, i.p.) and transcardially perfused with phosphate buffered saline (PBS) solution followed by 4.0% paraformaldehyde (PFA). Brains were postfixed overnight, cryoprotected in 30% sucrose and subsequently flash

frozen. Coronal sections (40 μm) through the entire hippocampus were collected onto slides in a 1:6 series and surviving cells and surviving newly born neurons were identified using immunohistochemistry for BrdU alone, and colocalization of BrdU and the neuronal marker NeuN, respectively. Briefly, sections were incubated in HCl (2M; 37°C, 45 mins), renatured in 0.1M sodium tetraborate (pH 8.5) and then blocked in 3% normal donkey serum (NDS; Sigma Cat# D9663). Sections were incubated with rat anti-BrdU antibody (Abcam Cat# AB6326; 1:250; overnight at 4°C), followed by AlexaFluor594 donkey anti-rat (Abcam Cat# ab150156; 1:500; 2 hours at room temperature) and mouse anti-NeuN (Millipore Cat# MAB377; 1:100; overnight at 4°C). Sections were then incubated in AlexaFluor488 donkey anti-mouse (Abcam Cat# ab150105 1:500; 2 hours at room temperature). To assess cell death, immunohistochemistry for the apoptosis marker caspase-3 was performed. Briefly, sections were incubated in H_2O_2 (in 1% methanol, 40 mins at room temperature) and then blocked in 10% normal goat serum (NGS; Sigma Cat# G9023). Sections were incubated with rabbit anti-active caspase-3 antibody (Promega Cat# G7481; 1:250; overnight at 4°C), followed by sequential incubations with the streptavidin-biotin immunoenzymatic antigen detection system (Abcam, Cat# ab64261). Images were obtained using an Olympus BX533 upright microscope coupled to an Olympus DP72 camera and Olympus FV1000 scanning laser confocal system (BioSciences Imaging Centre, UCC). Immunofluorescent Z-stack images with a 4.4 μm step size were collected using a 10x objective, while DAB staining was analysed at 20x magnification with bright field. Systematic random sampling was employed through the whole DG by counting the cells on both hemispheres of each section in 1:6 series (240 μm apart). Cell quantification was performed using the image processing

software package, ImageJ v 1.43m. All cell numbers are expressed as an average per section. The dorsal DG was defined as AP: - 0.94 to AP: - 2.30 and the ventral DG as AP: - 2.46 to AP: - 3.80 as described previously (O'Leary et al., 2012; Paizanis et al., 2010).

4.3 Results

Exercise significantly increased the number of surviving new cells in the whole DG of WT mice (exercise effect: $F_{1, 19} = 7.915$, $p = 0.011$), but not in Nr2e1$^{-/-}$ mice (exercise x genotype interaction: $F_{1, 19} = 6.747$, $p = 0.018$; Figure 4.2). Furthermore, both sedentary and running Nr2e1$^{-/-}$ mice exhibited a reduction in the number of surviving new cells compared to their WT littermates (genotype effect: $F_{1, 19} = 244.002$, $p < 0.001$; Figure 4.2). These changes in cell survival were driven by an exercise-induced increase in the number of surviving new neurons (neurogenesis) in the DG of WT mice (exercise effect: $F_{1,19} = 6.024$, $p = 0.024$), but not in Nr2e1$^{-/-}$ mice (exercise x genotype interaction: $F_{1,19} = 5.704$, $p = 0.027$; Figure 4.2). Additionally, sedentary Nr2e1$^{-/-}$ mice also exhibited reduced hippocampal neurogenesis compared to their WT littermates (genotype effect: $F_{1,19} = 130.096$, $p < 0.001$; Figure 4.2**)**. Upon subdivision of the DG into dorsal (dDG) and ventral (vDG) regions, this genotype effect was apparent in both subregions for both new cell (BrdU+) and new neuron (BrdU+NeuN+) survival (**BrdU+:** dDG: $F_{1,19} = 98.723$, $p < 0.001$; vDG: $F_{1,19} = 92.748$, $p < 0.001$; Figure 4.2; **BrdU+NeuN+:** dDG: $F_{1,19} = 69.635$, $p < 0.001$; vDG: $F_{1,19} = 88.056$, $p < 0.001$; Figure 4.2) . However, the exercise-induced increase in new cell survival and neurogenesis was only apparent in the dDG (exercise x genotype interaction: **BrdU+:** $F_{1,19} = 4.078$, $p = 0.058$; **BrdU+NeuN+:** $F_{1,19} = 4.502$; $p = 0.047$)

but not in the vDG of WT mice (Figure 4.2**)**. No difference was observed in the percentage of surviving new cells that adopted a neuronal fate in the DG of WT animals; almost all BrdU+ cells were immunopositive for NeuN and there was no effect of exercise on cells adopting a neuronal fate. In the DG of $Nr2e1^{-/-}$ mice, however, a significantly smaller percentage of surviving cells matured into neurons in both sedentary and exercise conditions (genotype effect: $F1,19 = 31.530$, $p < 0.001$; % BrdU+NeuN+ cells/BrdU+ cells: WT sedentary: M = 70.156, SD = 4.065; WT running: M = 74.398, SD = 9.338; Nr2e1-/- sedentary: M = 53.839, SD = 9.130; $Nr2e1^{-/-}$ running: M = 51.151, SD = 9.257).

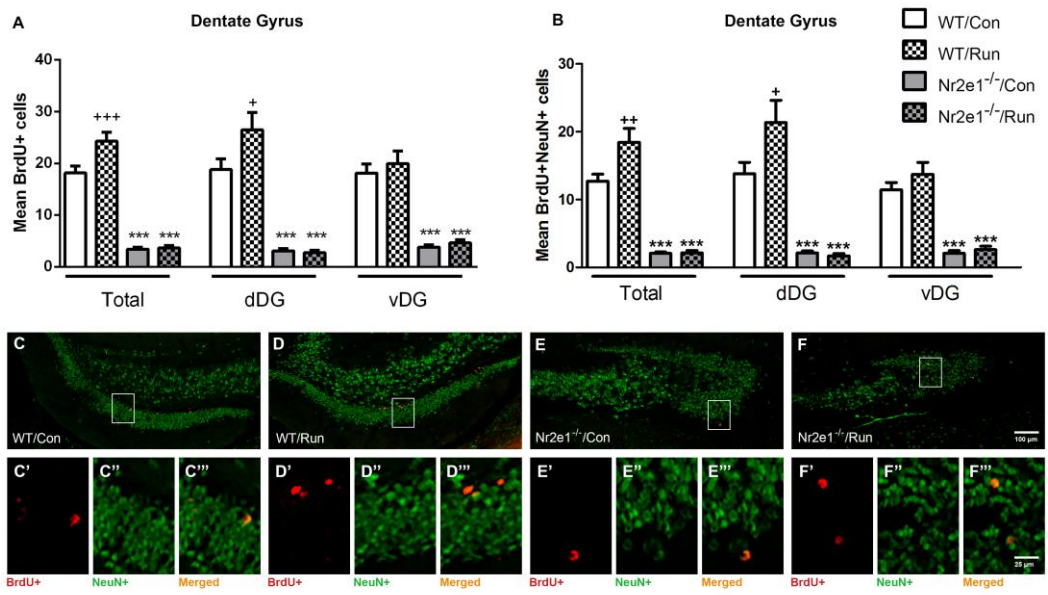

Figure 4.2: Nr2e1 is necessary for the pro-neurogenic effect of exercise to occur.
Mean number of BrdU+ (A) and BrdU+NeuN+ (B) cells per section in the whole, dorsal and ventral hippocampus of singly-housed wild type or $Nr2e1^{-/-}$ adolescent mice with or without access to running wheels. Data are expressed as mean ± SEM. *** $p < 0.001$ compared to WT counterparts; ++ $p < 0.01$, + $p < 0.05$ compared to WT sedentary mice; (Two-way ANOVA, Fisher's LSD), n = 5-6. Representative confocal images through the DG from WT sedentary (C) and running (D) and $Nr2e1^{-/-}$ sedentary (E) and running (F) mice. Immunohistochemical staining shows BrdU+ (red), NeuN+ (green) and BrdU+NeuN+ (orange) cells at 10X magnification. Scale bar = 100 µm. Higher magnification images depict immunopositive cells in the DG for BrdU (C' – F'), NeuN (C'' – F'') and merged channels (C''' – F'''). Scale bar = 25 µm.

Since exercise increased neurogenesis in the dorsal but not ventral DG of WT mice, we decided to investigate whether the stress of this social isolation during adolescence could explain the lack of effect of exercise-induced increases in neurogenesis in the vDG. Thus, we compared the effect of running on cell survival and hippocampal neurogenesis in single-housed compared to pair-housed WT mice (Figure 4.3). Two way ANOVA revealed that exercise significantly increased cell survival in paired- but not single-housed mice in the whole DG, and this effect persisted in both dDG and vDG (exercise x stress interaction: total DG: $F_{1,17} = 7.420$, $p = 0.014$; dDG: $F_{1,17} = 5.911$, $p = 0.026$; vDG: $F_{1,17} = 5.825$, $p = 0.027$; Figure 4.3). Interestingly, exercise significantly increased neurogenesis of both paired and single-housed mice who had access to a running wheel for three weeks compared to their sedentary counterparts (exercise effect: DG: $F_{1,17} = 34.054$; $p < 0.001$). However, the exercise-induced increase in neurogenesis was significantly lower in single-housed animals than in pair-housed animals (exercise x stress interaction: DG: $F_{1,17} = 6.866$; $p = 0.018$). Moreover, upon analysis of the dorsal and ventral regions of the DG, we found that although exercise increased neurogenesis in the dDG in both pair-housed and single-housed mice (dDG: $F_{1,17} = 29.350$; $p < 0.001$; Figure 4.3), this effect was attenuated by the stress of single housing (exercise x stress interaction dDG: $F_{1,17} = 5.239$, $p = 0.035$). Additionally, the exercise-induced increases in neurogenesis observed in the vDG of pair-housed mice (exercise effect: vDG: $F_{1,17} = 15.421$; $p = 0.001$) was prevented in singly housed mice (exercise x stress interaction: vDG: $F_{1,17} = 6.933$, $p = 0.017$; Figure 4.3).

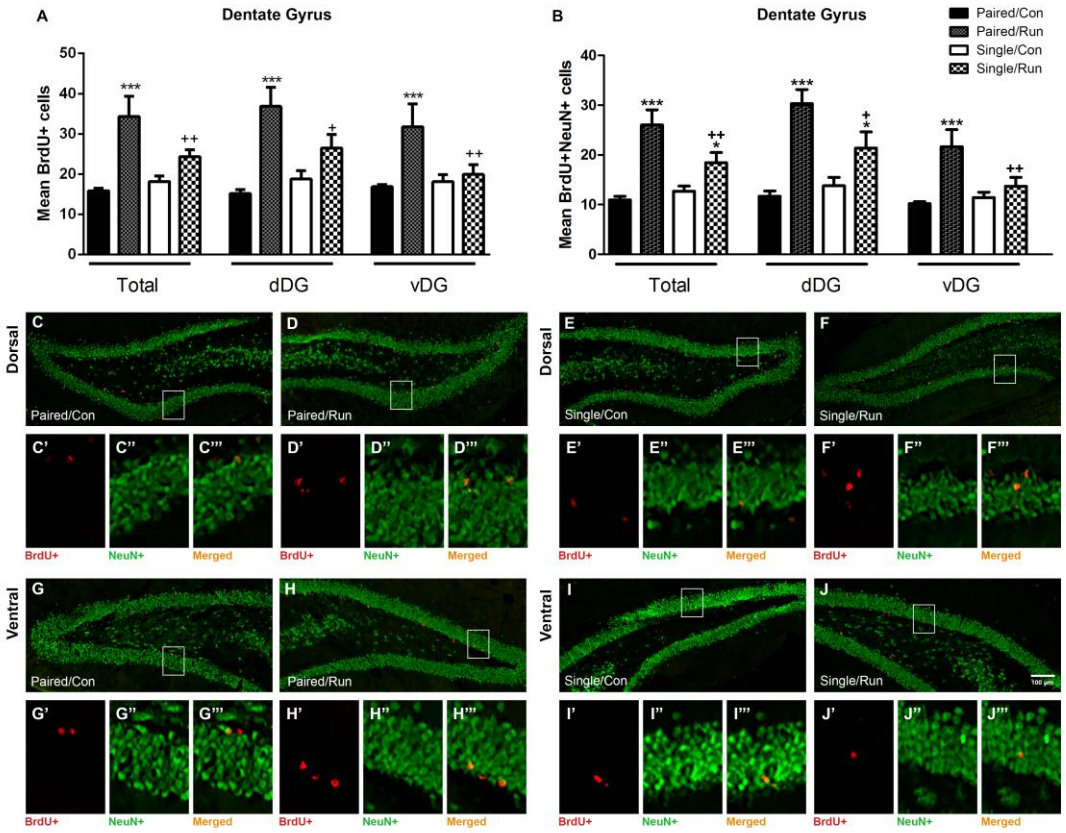

Figure 4.3: Differential modulation of neurogenesis by isolation stress and exercise across the septo-temporal axis of the DG in adolescent mice.
Mean number of BrdU+ (A) and BrdU+NeuN+ (B) cells per section in the whole, dorsal and ventral hippocampus of singly- or pair- housed adolescent mice with or without access to running wheels. Data are expressed as mean ± SEM. * $p < 0.05$, *** $p < 0.001$ compared to single- or pair- housed sedentary counterparts; + $p < 0.05$, ++ $p < 0.01$ compared to pair-housed running mice; (Two-way ANOVA, Fisher's LSD), n = 4-6. Representative confocal images of coronal sections through the dDG and vDG immunohistochemically stained with BrdU (red) and NeuN (green) from pair-housed sedentary (dDG: C, vDG: G), pair-housed running (dDG: D, vDG: H), single-housed sedentary (dDG: E, vDG: I) and single-housed running (dDG: F, vDG: J) mice. Images were taken at 10X magnification. Scale bar = 100 µm. Higher magnification images depict immunopositive cells in the DG for BrdU (dDG: C' – F', vDG: G' – J'), NeuN (dDG: C'' – F'', vDG: G'' – J'') and merged channels (dDG: C''' – F''', vDG: G''' – J'''). Scale bar = 25 µm.

There was a trend towards a reduction of caspase-3+ cells in the vDG of Nr2e1$^{-/-}$ mice with access to a running wheel, compared to their sedentary and WT running counterparts (exercise x genotype interaction: $F_{1,12} = 3.8$, $p = 0.077$; Figure 4.4**)**. There was a significant reduction in the number of apoptotic cells in the vDG but not dDG or whole DG in pair-housed mice with access to a running wheel compared to their

single-housed or pair-housed sedentary littermates (exercise x stress interaction: vDG: $F_{1,12} = 5.770$, $p < 0.05$; Figure 4.4).

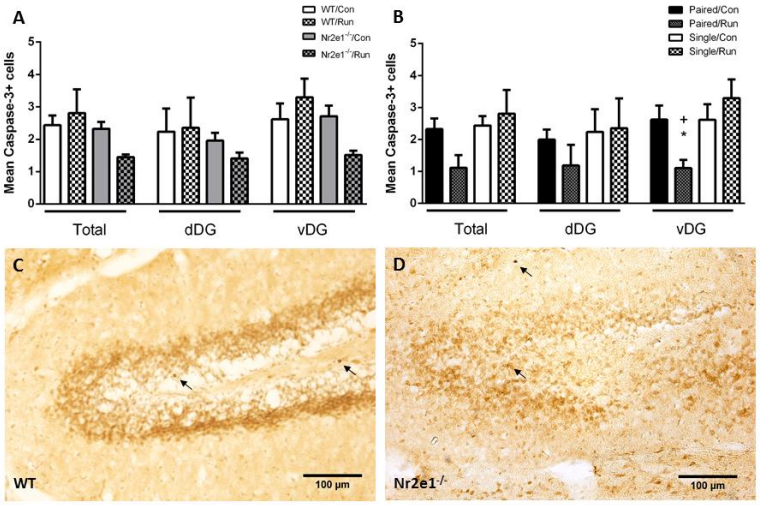

Figure 4.4: Differential modulation of apoptosis by isolation stress and exercise across the septotemporal axis of the DG in WT but not Nr2e1$^{-/-}$ adolescent mice.
Mean number of active caspase-3+ cells per section in whole, dorsal and ventral hippocampus of singly-housed wild type or Nr2e1$^{-/-}$ adolescent mice (A) and singly- or pair-housed wild type adolescent mice with or without access to running wheels (B). Data are expressed as mean ± SEM. * $p < 0.05$, compared to single- or pair- housed sedentary counterparts; + $p < 0.05$, compared to single-housed running mice; (Two-way ANOVA, Fisher's LSD), n = 4. Representative bright field images of coronal sections through the hippocampus immunocytochemically stained with active caspase-3 (dark brown; black arrows) from a wild type control (C) and Nr2e1$^{-/-}$ (D) mouse. Images were taken at 20X magnification. Scale bar = 100 µm.

4.4 Discussion

Our results indicate that the well-known pro-neurogenic effect of exercise observed during adulthood (van Praag et al., 1999a; van Praag et al., 1999b) also occurs in adolescent male mice. This finding corroborates the effects of exercise on cognitive function observed in adolescent rats (Hopkins et al., 2011). Mice exposed to exercise during adolescence have increased hippocampal levels of pro-neurogenic brain-derived neurotrophic factor (BDNF) (Gallego et al., 2015) and enhanced expression

of synaptic plasticity genes (Abel and Rissman, 2013). Exercise has also been shown to rescue alcohol-induced deficits in cell proliferation in adolescent rats (Helfer et al., 2009). Interestingly, a physical skills training task has recently been reported to increase cell survival in the DG of adolescent rats (DiFeo and Shors, 2017). However, training on this type of tasks involves physical exercise and learning, both of which increase neurogenesis and so a definitive conclusion on the effects of exercise alone during adolescence on hippocampal neurogenesis cannot be determined from this study. The present report is the first to demonstrate that voluntary running increases the survival of newly born cells and neurons in the DG of adolescent mice.

In the absence of TLX, a key regulator of adult neurogenesis, we found no pro-neurogenic effect of exercise. It has been previously shown that deletion of TLX leads to significant reduction in adult neurogenesis, synaptic plasticity and impaired dendritic structure in the DG of adult mice (Christie et al., 2006). Similarly, we show reduced survival of newborn cells and neurons in the DG of sedentary $Nr2e1^{-/-}$ adolescent mice, an effect which was not mitigated by exercise. This positions TLX as a regulator of exercise-induced increases in neurogenesis during adolescence. Further studies will determine whether TLX mitigates the pro-neurogenic effects of exercise in adulthood and indeed throughout the lifespan. We have previously shown that a lack of TLX is associated with impairments in hippocampal-related cognitive and anxiety behaviours during adolescence. Specifically, adolescent but not adult $Nr2e1^{-/-}$ mice showed deficits in spatial working memory, contextual fear conditioning and cued fear conditioning (O'Leary et al., 2016a). The fact that a lack of TLX

expression from birth through adulthood did not persistently induce the same impairments during adolescence and adulthood may point towards compensatory mechanisms occurring past the adolescent period, which ameliorate to some degree the deficits caused by deletion of TLX, independent of neurogenesis. It is worth noting that in contrast to our data with TLX deficiency, ablation of neurogenesis through irradiation, another model of reduced hippocampal neurogenesis, has been shown to be sensitive to the rescue effects of exercise (Clark et al., 2008; Naylor et al., 2008). However, the effects of adolescent hippocampal irradiation on neuronal survival remain unknown.

We observed an exercise-induced increase in neurogenesis, but not cell survival, in the DG of singly-housed adolescent mice which is consistent with previous reports using adult mice (Dostes et al., 2016; Gregoire et al., 2014; Kannangara et al., 2009). It is worth noting however that single housing has been shown to blunt cell proliferation in running rats (Leasure and Decker, 2009; Stranahan et al., 2006) but these differences have not been reconciled in adolescent rodents. To date, the effect of exercise and housing conditions on neurogenesis across the septo-temporal axis of the hippocampus has not been examined. This is interesting in light of the emerging view that the ventral hippocampus may be the predominant sub-region involved in stress responses (Bannerman et al., 2004; O'Leary and Cryan, 2014; Tanti and Belzung, 2013). Hence, the conflict in findings on hippocampal neurogenesis from group-housed and single-housed rodents may be a function of the differential effects of exercise on the dorsal and ventral hippocampus. Our results demonstrate that social

isolation prevents the pro-neurogenic effects of exercise in the vDG but not the dDG. Specifically, exercise increased the survival of new neurons in the dDG of both single- and pair-housed mice, but only in the vDG of pair-housed mice. In addition, the exercise-induced increase in neurogenesis in the dDG of both single and pair-housed mice was significantly attenuated by single housing. Importantly, the studies to date report on exercise-induced increases in neurogenesis in the whole DG, which may explain why studies employing single-housed mice consistently replicate the pro-neurogenic effects of exercise (Mustroph et al., 2012) observed in group-housed mice (van Praag et al., 1999a). Here we show that social isolation during adolescence acts as differential regulator of exercise across the distinct anatomical regions of the DG and propose that neurogenesis in both the dDG and vDG should be taken into consideration when investigating the role of hippocampal neurogenesis in exercise and stress-induced changes in behaviour. Nonetheless, the effects reported here may be specific to the adolescent period. Thus an examination of the effect of social isolation stress during other time periods of the lifespan on any potential exercise-induced changes in neurogenesis in the subregions of the DG is warranted in future studies.

The mechanisms underlying the differential effect of social isolation stress on exercise-induced changes in neurogenesis in the dDG and vDG during adolescence remain unclear. It has been reported that unpredictable chronic mild stress in adult mice preferentially decreased the survival of new neurons in the vDG (Elizalde et al., 2010; Tanti et al., 2012), supporting the view that neurogenesis in the ventral pole of the DG may be more susceptible to the effects of stress. Interestingly, environmental

enrichment, which includes exercise, promoted neurogenesis only in the dorsal hippocampus (Tanti et al., 2012). The effects of stress on neurogenesis are thought to be mediated by the glucocorticoid (GR) and mineralocorticoid (MR) receptors (Saaltink and Vreugdenhil, 2014), which are highly expressed in the hippocampus of rodents (Montaron et al., 2003). However, there are some preliminary and inconclusive findings regarding the difference in expression levels of the GR receptor in the dDG and vDG (Lin et al., 2012; Robertson et al., 2005), while MR receptor expression has been shown to be more concentrated in the vDG, at least in the rat brain (Robertson et al., 2005). Notwithstanding, both receptors have been shown to have a distinct activation pattern across the septo-temporal axis of the DG in response to acute stress (Caudal et al., 2014; Dorey et al., 2012). Moreover, a recent report has demonstrated that exercise increased GR expression in the hippocampus in single-housed but not pair-housed adult mice (Pan-Vazquez et al., 2015). Whether adolescent social isolation stress can affect the pro-neurogenic effect of exercise through the differential expression and activation of GR and MR in the vDG remains to be investigated. Another potential vDG-mediated mechanism underlying the attenuation of exercise-induced increase in neurogenesis by stress is through changes in the pro-neurogenic plasticity molecule BDNF (Berchtold et al., 2005; Chen and Russo-Neustadt, 2009; Ploughman et al., 2007; Tang et al., 2008). This is due to the fact that a stressful spatial navigation task has previously been shown to differentially affect the expression of BDNF in the dorsal (increased expression) and ventral (decreased expression) subregions of the DG (Hawley et al., 2012). The N-methyl-D-aspartate (NMDA) receptor activation pathway has also been implicated in both stress- and exercise-related changes in hippocampal neurogenesis. Specifically, exercise induced

a robust increase in the activation of NMDA receptor albeit in cortical mouse tissue (Dietrich et al., 2005) and NMDA receptors have been reported to operate downstream of the stress hormone, corticosterone to regulate hippocampal neurogenesis (Cameron et al., 1998). Given that there is a lower density of binding sites for NMDA receptors in the vDG compared to dDG (Pandis et al., 2006), it could be speculated that the two stimuli (isolation stress and exercise) compete for activation of the same pathway. Finally, isolation stress and exercise may also differentially impact upon cell death in the dorsal and ventral DG. In the current study, we show a significant decrease in the number of apoptotic cells in the vDG of pair housed mice with access to a running wheel. This may reflect a protective mechanism of exercise against cell death, which is attenuated by isolation-induced stress. However, it is important to note that the apoptosis measured here accounted for all cells in the vDG, hence we cannot conclude that the effects of isolation stress and exercise on apoptosis are specific to newly generated neurons.

It is surprising that we found no differences in the survival of newborn neurons of either area of the DG in single- and pair- housed sedentary adolescent mice. Studies conducted during adulthood have reported that social isolation results in anxiety- and depression-like behaviours in mice along with a reduction in levels of neuroplasticity genes (Berry et al., 2012; Ieraci et al., 2016). Social isolation during adolescence in non-human primates (marmosets) also impaired hippocampal neurogenesis in time-dependent manner (Cinini et al., 2014). Moreover, social isolation during adulthood has been shown to delay the pro-neurogenic effects of exercise in rats (Stranahan et

al., 2006). Interestingly, evidence from the Pereira lab suggests that an enriched environment is necessary to promote neurogenesis in single- housed adult mice (Monteiro et al., 2014). It is possible that in the absence of other external stimuli, social condition does not affect neurogenesis during the adolescent period, possibly due to the high basal rate of neurogenesis that occurs during adolescence compared to adulthood (He and Crews, 2007). Alternatively, the mouse strain used in the current study (generated on a BL6129 background) may have been a confounding factor by potentially limiting our ability to detect any downregulation of new neurons. In several independent studies examining the role of genetic influence on the baseline level of hippocampal neurogenesis, the B6129SF1 and 129Sv were among the strains showing the lowest levels of newborn neurons (Clark et al., 2011; Kempermann et al., 1997a; Merritt and Rhodes, 2015).

In conclusion, our results demonstrate that social isolation stress during adolescence attenuates an exercise-induced increase in neurogenesis. We show that this effect is most pronounced in the ventral hippocampus, a brain sub-region which plays a predominant role in anxiety and in regulating the stress response. Adolescence is a critical period for susceptibility to stress-related disorders as well as a time during which remodeling of hippocampal connectivity, including neurogenesis occurs. Thus, the impact of stress during adolescence on hippocampal neurogenesis and associated behaviours may be particularly potent. We also show that TLX is necessary for the pro-neurogenic effects of exercise during adolescence and have previously shown that the role of TLX in anxiety-related behaviours is most apparent during adolescence.

TLX is thus an important intrinsic regulator of exercise-induced changes in neurogenesis and may be a key target in understanding the interaction between positive and negative modifiable lifestyle factors such as stress and exercise on hippocampal neurogenesis and associated behaviours during adolescence.

Chapter 5

The Nuclear Receptor Tlx Regulates Motor, Cognitive and Anxiety-Related Behaviours during Adolescence and Adulthood

James D. O'Leary[a+], Danka A. Kozareva[a+], Cara M. Hueston[a], Olivia F. O'Leary[a], John F. Cryan[a,b] and Yvonne M. Nolan[a, b]

[a]Department of Anatomy and Neuroscience, University College Cork, Ireland

[b]APC Microbiome Institute, University College Cork, Ireland Department, University, Cork, Ireland

[+] Equal contribution

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Abstract

The nuclear receptor Tlx is a key regulator of embryonic and adult hippocampal neurogenesis and has been genetically linked to bipolar disorder. Mice lacking Tlx (*Nr2e1*$^{-/-}$) display deficits in adult hippocampal neurogenesis and behavioural abnormalities. However, whether Tlx regulates behaviour during adolescence or in a sex-dependent manner remains unexplored. Therefore, the role of Tlx was investigated in a series of behavioural tasks in adolescent male and female mice with a spontaneous deletion of Tlx (*Nr2e1*$^{-/-}$ mice). Testing commenced at adolescence (postnatal day 28) and continued until adulthood (postnatal day 67). Adolescent male and female *Nr2e1*$^{-/-}$ mice were hyperactive in an open field, an effect that persisted in adulthood. Male but not female *Nr2e1*$^{-/-}$ mice exhibited reduced thigmotaxis during adolescence and adulthood. Impairments in rotarod motor performance developed in male and female *Nr2e1*$^{-/-}$ mice at the onset of adulthood. Spontaneous alternation in the Y-maze, a hippocampus-dependent task, was impaired in adolescent but not adult male and female *Nr2e1*$^{-/-}$ mice. Contextual fear conditioning was impaired in adolescent male *Nr2e1*$^{-/-}$ mice only, but both male and female adolescent *Nr2e1*$^{-/-}$ mice showed impaired cued fear conditioning, a hippocampal-amygdala dependent cognitive process. These deficits persisted into adulthood in males but not females. In conclusion, deletion of Tlx impairs motor, cognitive and anxiety-related behaviours during adolescence and adulthood in male and female mice with most effects occurring during adolescence rather than adulthood independent of housing conditions. This suggests that Tlx has functions beyond regulation of adult hippocampal neurogenesis, and may be an important target in understanding neurobiological disorders.

5.1 Introduction

The orphan nuclear receptor Tlx, encoded by the gene *Nr2e1*, is a key regulator of embryonic and adult neurogenesis, with expression localized within the neurogenic niche of the forebrain and retina (Islam and Zhang, 2015; Monaghan et al., 1995; Shi et al., 2004). Tlx has been shown to be crucial for neural and retinal development (Li et al., 2008b; Miyawaki et al., 2004). Mice lacking Tlx display hypoplasia of the retina, cerebrum and olfactory bulbs as well as malformation of the limbic system, specifically the DG within the hippocampus (Miyawaki et al., 2004; Monaghan et al., 1997; Roy et al., 2002; Young et al., 2002; Yu et al., 2000). Moreover, deletion of Tlx has been shown to impair adult neurogenesis, synaptic plasticity and to negatively affect dendritic structure within the DG of adult mice (Christie et al., 2006). Thus, alterations in Tlx expression are likely to affect hippocampus-dependent behaviour.

Several different mouse models have been developed to target Tlx *in vivo*, such as targeted disruption by homologous recombination (Roy et al., 2002; Shi et al., 2004) , spontaneous deletion (Young et al., 2002) and conditional deletion (Zhang et al., 2008) (collectively referred to here as *Nr2e1*$^{-/-}$). Differences between these models make it difficult to draw comparisons. However, similarities are seen across models as mice with impairments in Tlx function have shown a number of behavioural abnormalities. The most striking behavioural phenotype of mice with a spontaneous deletion is aggression, which is regulated by the prefrontal cortex and limbic system (Rosell and Siever, 2015). This circuitry has previously been shown to be defective in *Nr2e1*$^{-/-}$ mice with both a targeted disruption of Tlx by homologous recombination and

spontaneous deletion (Monaghan et al., 1997; Young et al., 2002). Hyperactivity has also been documented in mice with a spontaneous deletion of Tlx from as early as postnatal day (P) 18 (Wong et al., 2010). Furthermore, impairments in spatial learning have been observed in adult mice with a conditional deletion of Tlx, while contextual and cued fear memory were unaffected (Zhang et al., 2008). Conversely, it has been shown that following a targeted disruption of Tlx by homologous recombination, mice exhibited poor contextual and cued fear recall, despite normal fear acquisition, in addition to reduced anxiety-like behaviour within the elevated plus maze (Roy et al., 2002). The reasons for the discrepancies across studies in adult mice are unclear but may be a function of the different methods used to reduce or inhibit Tlx expression, and/or when Tlx disruption occurs, such as early life or adulthood (Roy et al., 2002; Zhang et al., 2006). When the Tlx transgene was overexpressed using lentivirus-mediated means or in transgenic mice, an increase in adult hippocampal neurogenesis and enhanced performance in the Morris water maze as well as prepulse inhibition was observed (Murai et al., 2014). This work suggests a role for Tlx in learning and memory through its regulatory effect on adult hippocampal neurogenesis.

The mammalian brain continues to develop after birth, throughout childhood and into adulthood (Sisk and Foster, 2004; Spear, 2004). The adolescent period, which occurs in mice in postnatal weeks 3-8 (Laviola et al., 2003), is a critical developmental window when crucial neural circuits are established via a period of synaptic re-modelling (Andersen, 2003; Blakemore and Choudhury, 2006) and is a key period for susceptibility to stress and the emergence of neurobiological disorders such as

schizophrenia, depression and anxiety (Fuhrmann et al., 2015; Giedd et al., 2008) (Fuhrmann et al., 2015; Giedd et al., 2008; Green and Nolan, 2014; O'Connor and Cryan, 2014; Spear, 2007). Interestingly, linkage analysis studies of patients with bipolar disorder have reported susceptibility loci on chromosomes where *Nr2e1* is expressed (Kumar et al., 2008) thus suggesting a potential link between Tlx and mood disorders. Several studies have characterized the expression and the functional role of Tlx within the brain during embryonic and early postnatal development (Islam and Zhang, 2015; Monaghan et al., 1995; Roy et al., 2004; Roy et al., 2002; Wong et al., 2010). However, the functional role of Tlx during adolescence remains largely unexplored. In particular, it is not yet clear whether there are critical periods during postnatal life when Tlx might play a more dominant role in cognition, and whether such effects are sex-dependent. Thus, the aim of this study was to explore the extent and involvement of Tlx in hippocampus dependent cognition as well as hippocampus-independent functions during adolescence and adulthood in both male and female mice.

5.2 Materials and Methods

5.2.1 Experimental Design

Behavioural analysis was carried out in male and female mice with a spontaneous deletion of the Tlx gene (*Nr2e1*$^{-/-}$), heterozygous (*Nr2e1*$^{+/-}$) and wild type littermates. In order to capture potential deficits that may manifest during the adolescent developmental period, behavioural testing commenced at P28 and continued into adulthood until P67. Sensorimotor tests and motor performance tests on the rotarod

were conducted each week. Open field tests, spontaneous alternation in the Y-maze, and contextual and cued fear conditioning were conducted during adolescence (P28-35), and again in adulthood (P56-67; see Figure 5.1 for experimental design). *Nr2e1*$^{-/-}$ mice display impaired eye sight, therefore the behavioural tasks employed were chosen to minimize the dependency on visuospatial learning as much as possible (Brown and Wong, 2007; Dember and Roberts, 1958; Morgan et al., 2008).

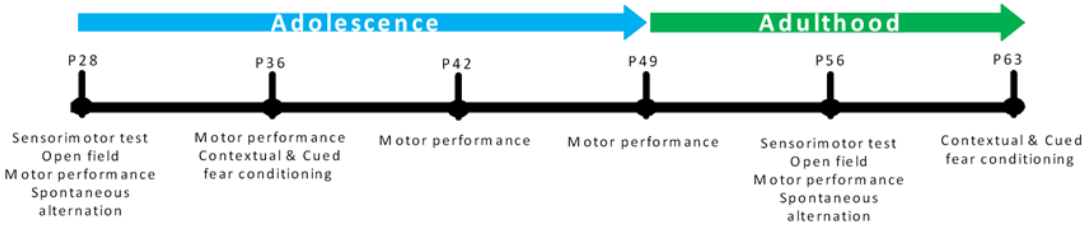

Figure 5.1: Experimental design. *Nr2e1*$^{-/-}$, *Nr2e1*$^{+/-}$ and wildtype mice were tested during adolescent development (P28 – P49) and adulthood (P56 – P67).

5.2.2 Animals

The animals used in the present study were all first generation offspring on a hybrid B6129 background resulting from mating male heterozygote (*Nr2e1*$^{+/-}$) mice on a 129S1/SvImJ background with female heterozygote (*Nr2e1*$^{+/-)}$ mice on a C57BL/6J background. They were kindly provided by Prof. Elizabeth Simpson, University of British Colombia and were generated as previously described (Wong et al., 2010). These mice exhibit a spontaneous deletion of the entire *Nr2e1* allele, including all nine exons. However, the deletion of the Tlx gene does not affect the transcription of neighbouring genes (Kumar et al., 2004). The impact of maternal care was controlled for as all animals were first generation littermate offspring resulting from mating male heterozygote (*Nr2e1*$^{+/-}$) mice with female heterozygote (*Nr2e1*$^{+/-}$) mice. All pups were weaned at P21. Due to the aggression that has been previously described in this strain

male *Nr2e1*$^{-/-}$ mice were singly housed after weaning (Young et al., 2002). Male wild type and heterozygous littermates and all female mice were grouped housed in standard housing conditions (temperature 21°C and relative humidity 55%). All mice had food and water available *ad libitum*. All experiments were conducted in accordance with the European Directive 2010/63/EU, and under an authorization issued by the Health Products Regulatory Authority Ireland and approved by the Animal Ethics Committee of University College Cork.

5.2.3 Body weight, growth rate and primary observation test

Animals were weighed, and growth rate calculated each week [(present weight – past weight)/ past weight x100]. Sensorimotor tests were conducted to identify any gross impairment which may have affected behavioural testing. The primary observation scores and sensorimotor testing were adapted from the Irwin behavioural screen (Crawley, 2007; Cryan et al., 2003; Irwin, 1968). This included measures of general health and physical appearance as well as sensorimotor reflexes, piloerection, palpebral closure, salivation, tremors, gait, trunk curl, pinna reflex, whisker reflex, reaching reflex, eye reflex, righting reflex, toe pinch, and provoked biting as a measure of aggression. Observations were recorded each week (P30, P37, P44, P51 and P58) and a score was assigned as indicated in Figure 5.2.

Physical Characteristics				Sensorimotor Reflexes			
Presence of Whiskers	**Score**	**Appearance of Fur**	**Score**	**Gait**	**Score**	**Trunk Curl**	**Score**
None	0	Ungroomed and disheveled	0	Normal	0	Absent	0
A few	1	Somewhat disheveled	1	Fluid but abnormal	1	Present	1
Most, but not a full set	2	Well-groomed (normal)	2	Limited movement only	2		
A full set	3			Incapacity	3	**Eye Reflex**	
		Wounds				Present	0
Piloerection		None	0	**Reaching Reflex**		Absent	1
None	0	Signs of previous wound	1	None	0		
Most hairs on end	1	Slight wounds present	2	Upon nose contact	1	**Whisker Reflex**	
		Moderate wounds present	3	Upon vibrissae contact	2	Present	0
Respiration		Extensive wounds present	4	Before vibrissae contact	3	Absent	1
Gasping, irregular	0			Early vigorous extension	4		
Slow, shallow	1	**Salivation**				**Toe Pinch**	
Normal	2	None	0	**Provoked Biting**		None	0
Hyperventilation	3	Slight margin of sub-maxillary area	1	Absent	0	Slight withdrawal	1
		Wet zone entire sub-maxillary area	2	Present	1	Moderate withdrawal, not brisk	2
Patches of Fur missing on Face						Brisk, rapid withdrawal	3
		Patches of Fur missing on body					
None	0			**Pinna Reflex**		Very brisk, repeated	4
Some	1	None	0	None	0	extension and flexion	
Extensive	2	Some	1	Active retraction, moderately brisk flick	1		
		Extensive	2	Hyperactive, repetitive flick	2		
Palpebral Closure							
Eyes wide open	0	**Skin Color**		**Righting Reflex**		**Tremor**	
Eyes 1/2 closed	1	Blanched	0	No impairment	0	None	0
Eyes closed	2	Pink	1	Number of sec required to right	1 to 10	Mild	1
		Bright, deep red, flushed	2			Marked	2

Figure 5.2: Primary observation test parameters. Adapter from (Irwin, 1968)

5.2.4 Locomotor activity and thigmotaxis in the open field

Spontaneous exploratory locomotor activity and thigmotaxis in the open field were used as a general measure of motor function and anxiety-related behaviours, respectively (Crawley, 2007). Animals were placed within a rectangular open field (32 x 40 cm; made in house) for 10 minutes. Locomotor activity is a simple measure of the distance the animal travels within the open field during the test, where large distances indicate hyperactivity. Thigmotaxis refers to the tendency of rodents to stay close to the walls of a maze during exploration (Choleris et al., 2001; Crawley, 2007). The behavioural test measures anxiogenesis induced by exposure to a novel environment as rodents tend to avoid open spaces and stay close to borders of maze arenas. Both locomotor activity and thigmotaxis were analysed using specialized software (Ethovision XT, Noldus Information Technology, USA).

5.2.5 Motor performance in the rotarod

Performance on the accelerating rotarod is a well-established measure of motor performance (Crawley, 2007) and was assessed in this paradigm using a protocol adapted from Menalled, El-Khodor (Menalled et al., 2009). The mice were placed on the rotarod apparatus (Ugo Basile, Italy) for five minutes and tested on an accelerating protocol (4 RPM to 40 RPM over five minutes, averaging 7.2 RPM acceleration). The latency for each mouse to fall was recorded. The mice were tested during three trials a day for three consecutive days (total nine trials), with the best score recorded. The test was repeated at weekly intervals beginning P28, P35, P42, P49 and P56, respectively. A reduced latency to fall indicates impairment in motor performance

and suggests a dysfunction within the cortical-striatal circuit which regulates motor behaviour.

5.2.6 Spontaneous alternation in the Y maze

Spontaneous alternation behaviour is the tendency of rodents to alternate their exploration of maze arms (such as those of the Y maze) and is used as a measure of hippocampal-dependent working memory as previously described (Hughes, 2004). The Y maze consisted of three arms 120° from each other (16 cm x 6.5 cm; made in house). The protocol was adapted from Senechal et al. (2007) (Senechal et al., 2007). Each animal was placed into the first arm of the maze facing the wall and allowed to explore the maze for five minutes. The number and order of arm entries were recorded. An arm entry was defined as all four paws entering into the arm (four paw criteria). An alternation was determined as the number of consecutive entries into the three maze arms. Alternations were then divided by the total number of entries during the five minute test period.

5.2.7 Contextual and cued fear conditioning

Contextual fear conditioning was used to assess hippocampal-dependent learning, while cued fear conditioning was employed to probe amygdala-dependent cognitive processes as previously described (Maren, 2001; Pattwell et al., 2011). During acquisition, animals were first placed into the fear conditioning chamber (Med Associates, 30.5 cm x 24.1 cm x 21.0 cm) which was scented with a lemon and ginger tea bag (Twinings™). Animals were allowed to explore the chamber for two minutes during an acclimation period and then received three shock and tone pairs (30 s tone;

5 kHz; 70 dB; 1 s foot shock; 0.65 mA DC current) separated by 30 second intervals. Animals were placed back in their home cage one minute after the final shock. Contextual fear memory was assessed 24 hours later by placing the animals back into the same chamber, but in the absence of tone and shock. Freezing behaviour (sec) was measured during the last 3.5 minutes of the total 5.5 minute protocol using specialized software (Video freeze, Med Associates, USA).

Cued fear conditioning was measured 24 hours after the contextual test in the same chambers. To measure cued fear learning, animals were placed into a novel context (white floor; black wall insert at 60°; and almond scent 1%) with presentation of the tone but no foot shock. Animals were allowed two minutes to acclimatize followed by three tone presentations (30 s; 5 kHz; 70 dB). Freezing behaviour during the 30 second tone presentations was recorded (Video freeze, Med Associates, USA). Contextual and cued fear conditioning was assessed during adolescence and adulthood with mice reconditioned to the tone and context in adulthood. Prior to reconditioning in adulthood, mice were placed back into the initial shock chamber to assess contextual fear memory retention. Twenty four hours later mice were placed in the same chamber as the cued fear conditioning chamber in order to assess cued fear memory recall retention.

5.2.8 Statistical analysis

All data were analysed using SPSS statistical software (SPSS, Chicago, IL). Data from body weight, rotarod motor performance, and cued fear conditioning were analysed

by repeated measures ANOVA with Bonferroni post hoc test. Data from open field, spontaneous alternation and contextual fear conditioning were analysed by one-way ANOVA, with Fisher's LSD post hoc analysis. Nonparametric data from sensorimotor tests were analysed by the Kruskal–Wallis one-way ANOVA. An alpha level of 0.05 was used as criterion for statistical significance. Parametric data are presented as mean ± SEM. Nonparametric data are presented as percentage (%) displaying normal response.

5.3 Results

5.3.1 *Nr2e1*$^{-/-}$ mice have reduced body weight and increased growth rate during adolescence

Male and female mice gained weight throughout development (Figure 5.3). The Mauchly's test indicated that in the male cohort the assumptions of sphericity had been violated, (χ^2 (9) = 51.29, $p < 0.01$). Therefore, the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity as the epsilon was less than 0.75 ($\varepsilon = 0.595$). The results show that all male mice gain body weight with age (F (2.38, 83.32) = 421.39, $p < 0.01$; Figure 5.3). Female mice also showed a similar result, with all genotypes gaining weight with age (F (4, 136) = 253.83, $p < 0.01$; Figure 5.3). There was a significant effect of genotype on body weight throughout adolescence and adulthood, in both male (F (2, 35) = 17.74, $p < 0.01$) and female (F (2, 34) = 20.35, $p < 0.01$) mice, with *Nr2e1*$^{-/-}$ mice remaining significantly lighter than their wild type and heterozygous littermates (Bonferroni post hoc comparison, $p < 0.01$). There was also a significant interaction between age and genotype in male (F (4.76, 83.32) =

4.40, $p < 0.01$) and female (F (8, 136) = 3.21, $p < 0.01$) mice, indicating that *Nr2e1*$^{-/-}$ mice do not gain weight similarly to their wild type and *Nr2e1*$^{+/-}$ littermates.

Male and female mice show a reduction in the rate of growth as they approach adulthood (Figure 5.3). Interestingly, *Nr2e1*$^{-/-}$ mice appear to have a higher rate of growth during early adolescence (P35) compared to wild type and *Nr2e1*$^{+/-}$ littermates in both males (F (2, 35) = 18.69, $p < 0.01$) and females (F (2, 34) = 24.66, $p < 0.01$). Furthermore, this increased growth rate appears to normalize at the onset of adulthood (P49-56). These results indicate that while *Nr2e1*$^{-/-}$ mice gain body weight at a greater rate throughout development, body weight remains reduced compared to wild type and heterozygous littermates. This finding is stable across sex and is independent of housing conditions.

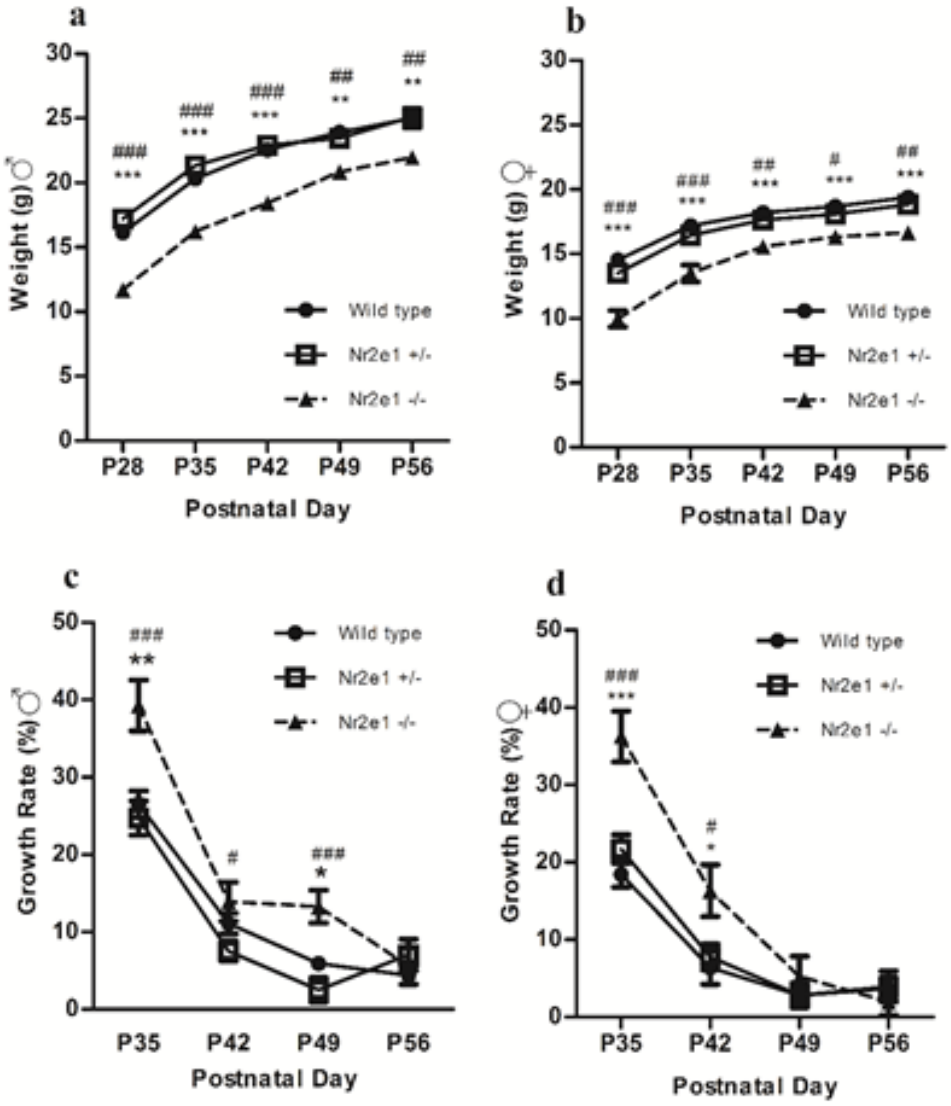

Figure 5.3: Body weight as a function of genotype, sex and age. Body weight of male (a) and female (b) mice; growth rate of male (c) and female (d) mice. ***p < 0.0001, **p < 0.001, *p < 0.05, $Nr2e1^{-/-}$ compared to wildtype mice. $^{###}p < 0.0001$, $^{##}p < 0.001$, $^{#}p < 0.05$, $Nr2e1^{-/-}$ compared to $Nr2e1^{+/-}$ mice; ANOVA with post hoc Bonferroni analysis. All results are expressed as mean ±SEM. Sample size per sex: wildtype (n=13-14), $Nr2e1^{+/-}$ (n=16), $Nr2e1^{-/-}$ (n=8).

5.3.2 $Nr2e1^{-/-}$ mice exhibit increased provoked biting and impaired eye reflex, reaching reflex and piloerection in a primary observation test battery

The results of the primary observation tests are summarised in Figure 5.4 and Figure 5.5. There was no significant difference across genotype, sex or age in animal

appearance (presence of whiskers, appearance of fur, and patches of fur missing, skin colour), respiration, tremors, salivation, gait, trunk curl, pinna reflex, whisker reflex, toe pinch reflex or righting reflex. However, both male and female *Nr2e1*$^{-/-}$ mice showed an increase in provoked biting during adolescence (male, $p < 0.05$ and female, $p < 0.05$; Figure 5.4). This increase in provoked biting continued in adulthood (Figure 5.5) but did not reach statistical significance (male, $p = 0.14$ and female, $p = 0.13$). Male *Nr2e1*$^{-/-}$ mice also exhibited impaired eye reflex during adolescence ($p < 0.05$; Figure 5.4). However, this wasn't observed during adulthood ($p > 0.05$; Figure 5.5). In addition, adolescent male *Nr2e1*$^{-/-}$ mice showed a trend towards impaired reaching reflex ($p = 0.057$; Figure 5.4). This pattern continued during adulthood in male mice but did not reach statistical significance (males $p = 0.12$; females $p > 0.05$; Figure 5.5). Further, adult male *Nr2e1*$^{-/-}$ mice exhibited an increase in piloerection ($p < 0.01$) and an increase in palpebral closure ($p < 0.05$; Figure 5.5).

Physical Characteristics	Male			Female		
	Wild type	Nr2e1$^{+/-}$	Nr2e1$^{-/-}$	Wild type	Nr2e1$^{+/-}$	Nr2e1$^{-/-}$
Presence of Whiskers (%)	100	100	100	100	100	100
Well-groomed fur (%)	100	100	100	100	100	100
Piloerection (%)	0	0	0	0	0	0
Missing fur on Face (%)	21.4	13.3	0	0	12.5	12.5
Missing fur on body (%)	0	0	12.5	7.6	18.7	12.5
Palpebral Closure (%)	0	0	0	0	0	0
Wounds (%)	0	6.25	0	0	6.2	0
Respiration	Normal	Normal	Normal	Normal	Normal	Normal
Tremor (%)	0	0	0	0	0	0
Skin Color	Normal	Normal	Normal	Normal	Normal	Normal
Salivation (%)	0	0	0	0	0	0
Sensorimotor Reflexes (% displaying normal response)						
Gait	100	100	100	100	100	100
Trunk Curl	100	100	100	92.3	93.7	100
Reaching Reflex	71.4	100	62.5	92.3	87.5	62.5
Pinna Reflex	85.7	87.5	75	100	75	87.5
Eye Reflex	**85.7**	**100**	**62.5***	90.09	66.67	100
Whisker Reflex	85.7	92.85	75	100	80	100
Toe Pinch	78.5	81.25	62.25	100	81.25	100
Righting Reflex (% impaired)	0	0	0	0	0	0
Provoked Biting (%)	**38.4**	**31.2**	**87.5***	**30.7**	**37.5**	**77.8***

Figure 5.4: Primary observation of Nr2e1$^{-/-}$ mice during adolescence. *$p < 0.05$, Nr2e1$^{-/-}$ compared to wildtype mice. Kruskal-Wallis one-way ANOVA. Wildtype (n=13-14), Nr2e1$^{+/-}$ (n=16), Nr2e1$^{-/-}$ (n=8).

Physical Characteristics	Male Wild type	Nr2e1$^{+/-}$	Nr2e1$^{-/-}$
Presence of Whiskers (%)	100	100	100
Well-groomed fur (%)	76.9	75	62.5
Piloerection (%)	**0**	**0**	**62.5*** **
Missing fur on Face (%)	0	0	0
Missing fur on body (%)	0	0	0
Palpebral Closure (%)	**0**	**0**	**25***
Wounds (%)	7.7	12.5	37.5
Respiration	Normal	Normal	Normal
Tremor (%)	0	0	0
Skin Color	100	100	100
Salivation (%)	7.7	0	33.33
Sensorimotor Reflexes (% displaying normal response)			
Gait	100	100	100
Trunk Curl	100	100	100
Reaching Reflex	92.3	62.5	50
Pinna Reflex	76.9	75	75
Eye Reflex	92.3	81.25	100
Whisker Reflex	84.61	87.5	100
Toe Pinch	100	93.7	100
Righting Reflex (% impaired)	0	0	0
Provoked Biting (%)	46.1	50	87.5

Physical Characteristics	Female Wild type	Nr2e1$^{+/-}$	Nr2e1$^{-/-}$
Presence of Whiskers (%)	84.6	81.2	87.5
Well-groomed fur (%)	100	93.7	100
Piloerection (%)	0	0	0
Missing fur on Face (%)	0	0	0
Missing fur on body (%)	0	0	0
Palpebral Closure (%)	0	0	12.5
Wounds (%)	0	0	12.5
Respiration	Normal	Normal	Normal
Tremor (%)	0	0	0
Skin Color	100	100	100
Salivation (%)	0	13.3	0
Sensorimotor Reflexes (% displaying normal response)			
Gait	100	100	100
Trunk Curl	84.6	93.7	100
Reaching Reflex	92.3	87.5	62.5
Pinna Reflex	100	62.5	87.5
Eye Reflex	100	86.66	83.33
Whisker Reflex	77.77	86.66	100
Toe Pinch	92.3	93.7	100
Righting Reflex (% impaired)	0	0	0
Provoked Biting (%)	46.1	31.2	75

Figure 5.5: Primary observation of Nr2e1$^{-/-}$ mice during adulthood. ***p < 0.0001, **p < 0.001, *p < 0.05, Nr2e1$^{-/-}$ compared to wildtype mice. Kruskal-Wallis one-way ANOVA. Wildtype (n=13), Nr2e1$^{+/-}$ (n=16), Nr2e1$^{-/-}$ (n=8).

5.3.3 Nr2e1$^{-/-}$ mice exhibit hyperactivity and deficits in cortico-striatal associated behaviour

5.3.3.1 Locomotor activity in the open field

Testing in the open field revealed that male (Figure 5.6) and female (Figure 5.6) *Nr2e1*$^{-/-}$ mice were hyperactive during adolescence (male F (2, 37) = 25.21, $p < 0.01$, female F (2, 36) = 18.05, $p < 0.01$), independent of housing conditions. Hyperactivity continued into adulthood in both male (F (2, 37) = 37.79, $p < 0.01$) and female (F (2, 36) 19.77, $p < 0.01$) *Nr2e1*$^{-/-}$ mice (Figure 5.6). Furthermore, hyperactivity appeared to be more pronounced during adulthood in both male and female *Nr2e1*$^{-/-}$ mice with an approximately threefold increase in distance travelled compared to wild types (Figure 5.6).

5.3.3.2 Thigmotaxis in the open field

Adolescent male *Nr2e1*$^{-/-}$ mice exhibited a significant increase in exploration of the centre of the open field (F (2, 37) = 3.90, $p < 0.05$) indicating a reduction in thigmotaxis behaviour (Figure 5.6). This observation continued into adulthood (F (2, 37) = 6.37, $p < 0.01$; Figure 5.6). All female mice showed similar thigmotaxis behaviour throughout adolescence (F (2, 36) = 0.46, $p > 0.05$) and adulthood (F (2, 36) = 1.39, $p > 0.05$; Figure 5.6).

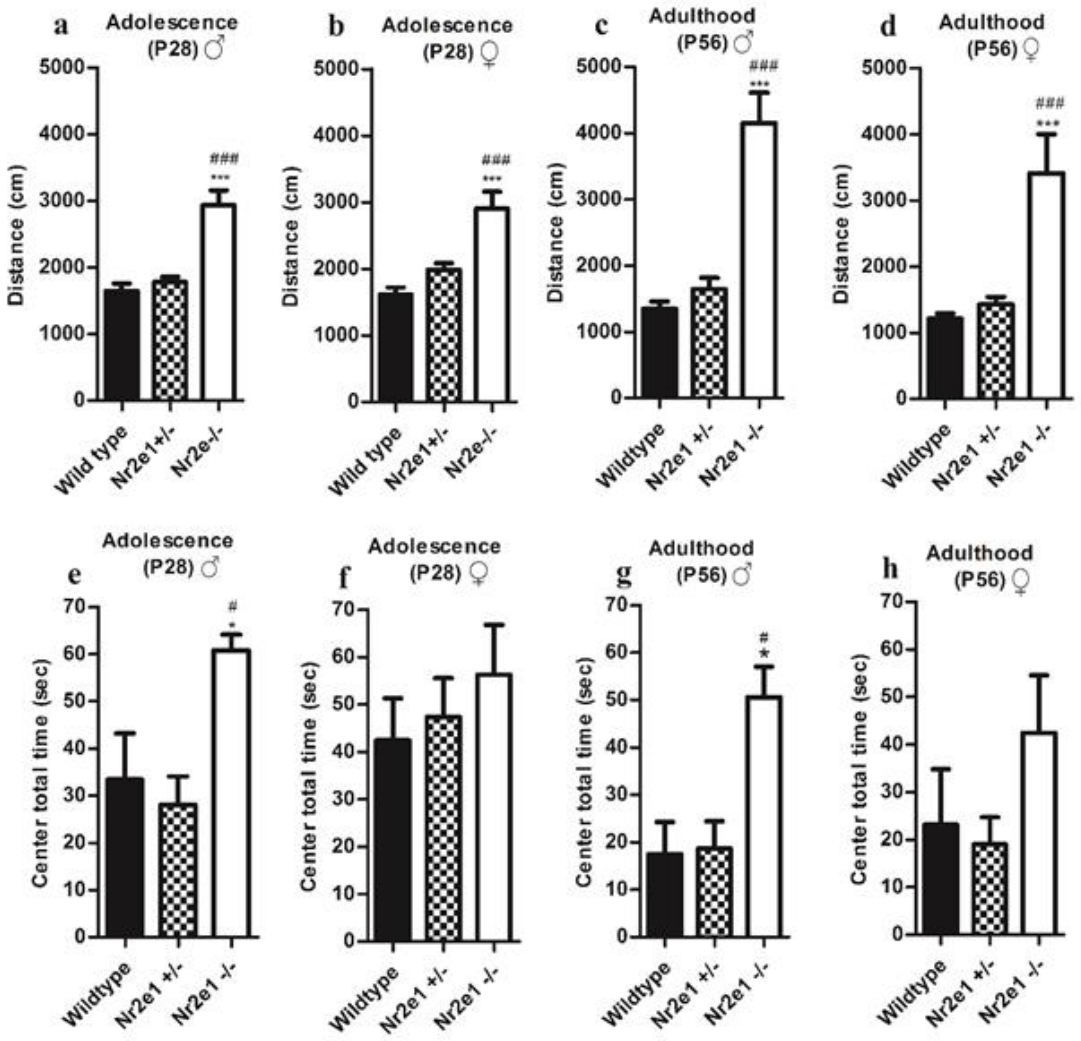

Figure 5.6: Locomotor activity and thigmotaxis in an open field as a function of genotype. Locomotor activity in adolescent male (a) and female (b) mice, and in adult male (c) and female (d) mice. Exploration of arena centre in adolescent male (e) and female (f) mice, and adult male (g) and female (h) mice. ***$p < 0.0001$, **$p < 0.001$, *$p < 0.05$, $Nr2e1^{-/-}$ compared to wildtype mice. $^{###}p < 0.0001$, $^{##}p < 0.001$, $^{#}p < 0.05$, $Nr2e1^{-/-}$ compared to $Nr2e1^{+/-}$ mice; ANOVA with post hoc Bonferroni analysis. All results are expressed as mean ±SEM. Sample size per sex: wildtype (n=13-14), $Nr2e1^{+/-}$ (n=16), $Nr2e1^{-/-}$ (n=8).

5.3.3.3 Motor performance on the rotarod

The Mauchly's test indicated the assumptions of sphericity had been violated in both male (χ^2 (9) = 27.64, $p < 0.01$), and female (χ^2 (9) = 18.77, $p < 0.027$) mice. Therefore, the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity for males as the epsilon was less than 0.75; ($\varepsilon = 0.74$), and the Huynh-Feidt estimates of sphericity for females as the epsilon was greater than 0.75, ($\varepsilon = 0.949$).

With this correction testing on the rotarod revealed that impairments in motor performance developed at the onset of adulthood (P42; Figure 5.7), in both male (F (2.69, 103.603) = 4.54, $p < 0.01$) and female (F (3.795, 129.026) = 9.36, $p < 0.01$) *Nr2e1*$^{-/-}$ mice, independent of housing conditions. There was a significant effect of genotype on motor performance, in both male (F (2, 35) = 6.88, $p < 0.01$; Figure 5.7) and female (F (2, 34) = 7.59, $p < 0.01$; Figure 5.7) *Nr2e1*$^{-/-}$ mice. There was also a significant interaction between age and genotype in male (F (5.92, 103.603) = 3.344, $p < 0.01$) and female (F (7.59, 109.202) = 2.97, $p <0.01$) mice. Bonferroni post hoc comparison revealed that impairments in motor performance developed in both male and female *Nr2e1*$^{-/-}$ mice at the onset of adulthood (Figure 5.7). This indicates that motor performance does not remain stable throughout development for *Nr2e1*$^{-/-}$ mice.

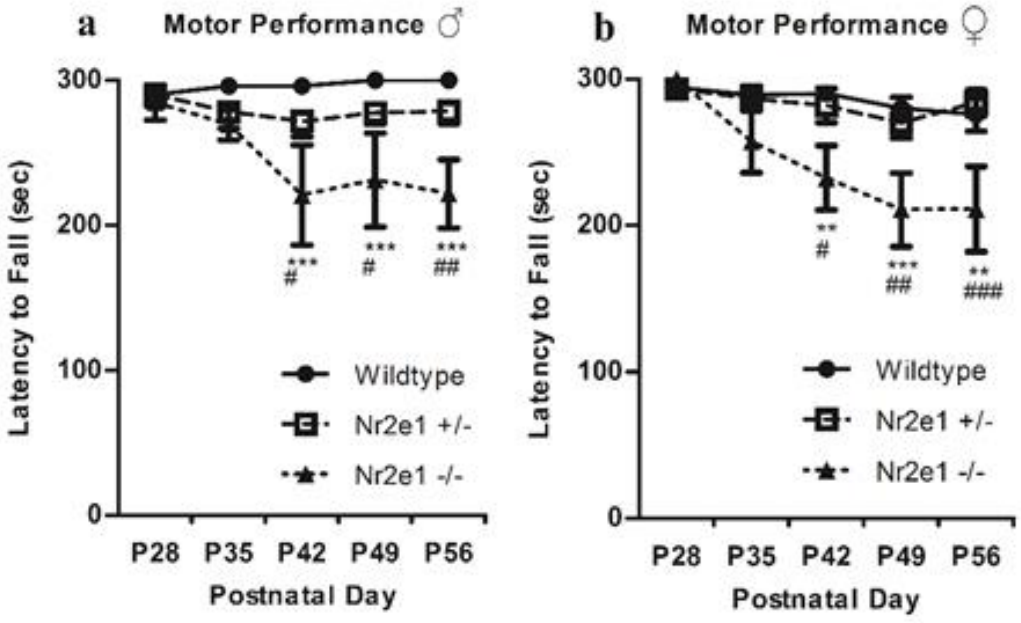

Figure 5.7: Motor performance in a rotarod latency to fall paradigm as a function of genotype. Motor performance on the rotarod in male (a) and female (b) mice. ***p < 0.0001, **p < 0.001, *p < 0.05, Nr2e1$^{-/-}$ compared to wildtype mice. $^{###}$p < 0.0001, $^{##}$p < 0.001, $^{#}$p < 0.05, Nr2e1$^{-/-}$ compared to Nr2e1$^{+/-}$ mice; ANOVA with post hoc Bonferroni analysis. All results are expressed as mean ±SEM. Sample size per sex: wildtype (n=13-14), Nr2e1$^{+/-}$ (n=16), Nr2e1$^{-/-}$ (n=8).

5.3.4 Nr2e1$^{-/-}$ mice exhibit deficits in hippocampal-associated cognition

5.3.4.1 Spontaneous alternation in the Y maze

In the spontaneous alternation test of working memory, male and female *Nr2e1*$^{-/-}$ mice showed impaired spontaneous alternation during adolescence compared to *Nr2e1*$^{+/-}$ and wild type mice, (male F (2, 37) = 4.60, $p < 0.01$ and female F (2, 36) = 3.97, $p < 0.05$; Figure 5.8). However, this effect did not persist into adulthood (Figure 5.8). A significant effect was observed in spontaneous alternation in adult male mice (F (2, 37) = 7.31, $p < 0.01$; Figure 5.8). Post hoc comparison using the Fisher's LSD test revealed this was due to an increase in *Nr2e1*$^{+/-}$ performance compared to wild type, ($p < 0.01$) and *Nr2e1*$^{-/-}$ mice, ($p < 0.01$). Female mice exhibited no overall difference in spontaneous alternation during adulthood (F (2, 36) = 1.80, $p > 0.05$; Figure 5.8).

5.3.4.2 Contextual fear conditioning

Male *Nr2e1*$^{-/-}$ mice showed impaired freezing behaviour during adolescence compared to *Nr2e1*$^{+/-}$ and wild type mice (F (2, 36) = 8.82, $p < 0.01$; Figure 5.8). Adolescent female *Nr2e1*$^{-/-}$ mice showed a trend for reduced contextual freezing but this did not reach statistical significance (F (2, 34) = 2.46, $p = 0.10$; Figure 5.8). Contextual freezing during adulthood did not differ across sex or genotype (male F (2, 36) = 2.34, $p > 0.05$; Figure 5.8); female (F (2, 28) = 0.47, $p > 0.05$; Figure 5.8).

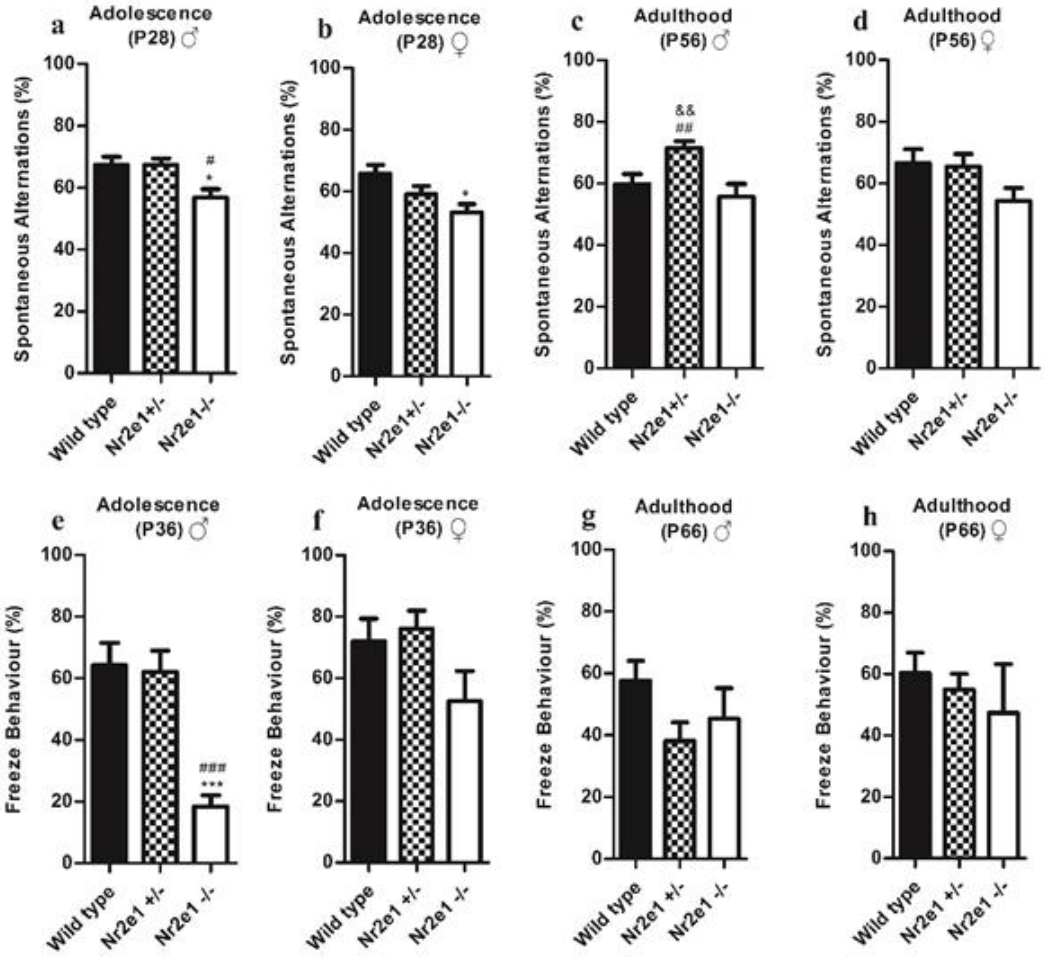

Figure 5.8: Spontaneous alternation (%) in the Y-maze and contextual fea conditioning as a function of genotype during adolescence (P28-36) and adulthood (P56-66). Spontaneous alternation in adolescent male (a) and female (b) mice, and adult male (c) and female (d) mice. Contextual freeze behaviour in adolescent male (e) and female (f) mice, and in adult male (g) and female (h) mice. . ***p < 0.0001, **p < 0.001, *p < 0.05, $Nr2e1^{-/-}$ compared to wildtype mice. $^{###}p < 0.0001$, $^{##}p < 0.001$, $^{#}p < 0.05$, $Nr2e1^{-/-}$ compared to $Nr2e1^{+/-}$ mice; ANOVA with post hoc Fisher's LSD test. All results are expressed as mean ±SEM. Sample size per sex: wildtype (n=8-14), $Nr2e1^{+/-}$ (n=15-16), $Nr2e1^{-/-}$ (n=6-8).

5.3.5 $Nr2e1^{-/-}$ mice show impaired hippocampal-amygdala dependent cognition

5.3.5.1 Cued fear conditioning

Both male and female *Nr2e1*$^{-/-}$ mice showed impaired cued fear recall during adolescence (male F (2, 34) = 3.62, $p < 0.05$ and female F (2, 32) = 10.17, $p < 0.01$; Figure 5.9). In adulthood, only male *Nr2e1*$^{-/-}$ mice exhibited impaired cued fear recall

(F (2, 34) = 14.31, $p < 0.01$; Figure 5.9). Interestingly, male *Nr2e1*$^{+/-}$ mice also exhibited impaired cued fear recall during adulthood, but not during adolescence. However, no impairment was observed in female *Nr2e1*$^{+/-}$ or *Nr2e1*$^{-/-}$ mice during adulthood (F (2, 26), = 0.96, $p > 0.05$; Figure 5.9).

To measure the retention of the cued fear memory that was acquired during adolescence, we first measured freezing behaviour in response to the cue but prior to the re-introduction of the unconditioned stimulus at P62 (Figure 5.9). In this cued fear memory retention test both male and female adult (P62) *Nr2e1*$^{-/-}$ mice exhibited poor retention of the fear memory that was acquired in adolescence (male F (2, 34) = 19.95, $p < 0.01$ and female F (2, 29) = 17.0, $p < 0.01$).

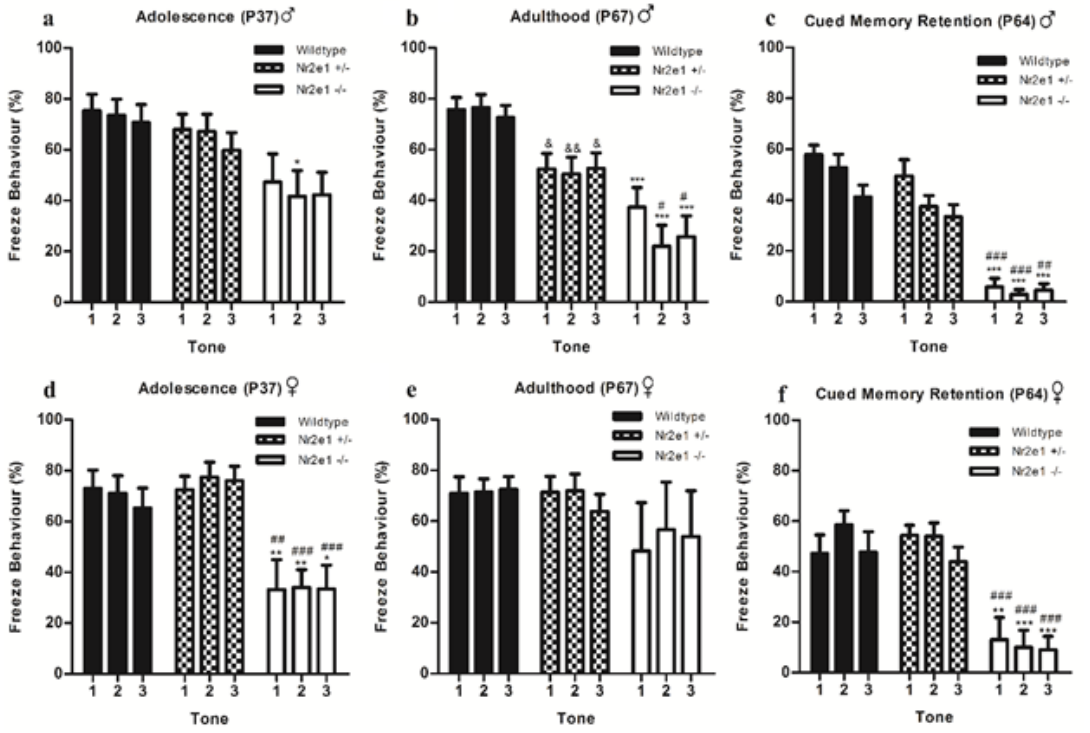

Figure 5.9: Cued fear conditioning as a function of genotype during adolescence (P37) and adulthood (P67). Cued fear conditioning in adolescent male (e) and female (d) mice, and in adult male (c) and female (f) mice. Cued fear retention test in male (b) and female (e) mice. ***$p < 0.0001$, **$p < 0.001$, *$p < 0.05$, $Nr2e1^{-/-}$ compared to wildtype mice. $^{###}p < 0.0001$, $^{##}p < 0.001$, $^{#}p < 0.05$, $Nr2e1^{-/-}$ compared to $Nr2e1^{+/-}$ mice; $^{&}p < 0.05$, $Nr2e1^{+/-}$ compared to wildtype mice. ANOVA with post hoc Bonferroni analysis. All results are expressed as mean ±SEM. Sample size per sex: wildtype (n=8-14), $Nr2e1^{+/-}$ (n=13-16), $Nr2e1^{-/-}$ (n=6-8).

5.4 Discussion

This study demonstrated that Tlx has a role to play in motor, cognitive and anxiety-related behaviour during adolescence and adulthood independent of sex or housing conditions, with most impact during adolescence. Both adolescent male and female *Nr2e1*$^{-/-}$ mice showed deficits in spatial working memory as measured by spontaneous alternation in the Y maze. Further, adolescent male but not female *Nr2e1*$^{-/-}$ mice showed deficits in hippocampal function as measured by contextual fear conditioning but these effects in hippocampus-dependent memory tasks did not persist into adulthood. Previous studies have reported contradictory findings regarding the

involvement of Tlx in hippocampus-associated cognition in adult mice. Similar to the present study, it has been shown that normal fear acquisition and contextual fear conditioning occurs in adult male *Nr2e1*$^{-/-}$ mice (Zhang et al., 2008). Impaired associative fear memory in contextual fear conditioning in adult male mice with a targeted disruption of Tlx has also been reported (Roy et al., 2002). The present study is to our knowledge the first report of impaired spatial working memory in adolescent male and female *Nr2e1*$^{-/-}$ mice with a spontaneous deletion; however, this deficit did not persist into adulthood. This is in contrast to the previously reported impairments in spatial working memory in Tlx deficit mice albeit using the Morris water maze and in mice with a conditional deletion in adulthood (Zhang et al., 2008), rather than a spontaneous deletion. The reasons for the discrepancies across studies in adult mice are unclear but may be a function of the different methods used to reduce or inhibit Tlx expression, or the time at which the Tlx disruption occurs. Zhang et al. (2008) generated a conditional deletion of Tlx in adult mice localized to the forebrain and olfactory bulbs, whereas Roy et al. (2002) generated a transgenic strain with a targeted disruption of Tlx (Roy et al., 2002; Zhang et al., 2008). In the mice used in the present study, the Tlx deletion occurs from birth via a spontaneous deletion of all nine exons of the gene. It is important to consider that germline mutation models such as the *Nr2e1*$^{-/-}$ mice used in the current study and by others (where Tlx disruption occurs in early life) may impact upon developmental processes (Shi et al., 2004; Wong et al., 2010; Young et al., 2002) that could thus contribute to the behavioural phenotype. Indeed, early life appears to be a sensitive period to Tlx disruption as indicated by greater neuroanatomical and behavioural impairments in mice with an early life deletion of Tlx compared to mice with an adult knockdown (Shi et al., 2004; Wong et

al., 2010; Young et al., 2002; Zhang et al., 2008). Thus, the method of interference on Tlx expression may affect the level of impairment observed, and in turn might at least partially explain the inconsistent findings. Taken together, while there is evidence that Tlx plays a role in hippocampus-dependent cognition, adolescence may be the more susceptible period to disruption of spatial working memory and hippocampal processes from Tlx deletion.

During adolescence, both male and female *Nr2e1*$^{-/-}$ mice exhibited impaired cued fear conditioning, a hippocampal-amygdala dependent cognitive process (Maren, 2001). Interestingly, these deficits persist and are more pronounced in adult male *Nr2e1*$^{-/-}$ mice. On the other hand, the cued fear memory impairments observed in adolescent female *Nr2e1*$^{-/-}$ mice did not persist into adulthood. While *Nr2e1*$^{-/-}$ mice exhibit some delay in cued fear acquisition, freezing behaviour reaches levels exhibited by wild type control mice by the end of the training period (data not shown) and so these impairments are not due to deficits in acquisition. Interestingly, we report the novel finding that male but not female *Nr2e1*$^{+/-}$ mice also exhibited impaired cued fear conditioning during adulthood. Previous studies have reported contradictory findings on cued fear conditioning in male *Nr2e1*$^{-/-}$ mice, with either normal (Zhang et al., 2008) or impaired (Roy et al., 2002) cued fear conditioning in male mice being reported. Unlike the present study however, the effects in female mice were not investigated in these earlier studies (Roy et al., 2002; Zhang et al., 2008). The reasons underlying the discrepancies in adult male *Nr2e1*$^{-/-}$ mice cued fear conditioning are not clear but may again be a function of the methods used to reduce or inhibit Tlx

expression or possibly the experimental variables in the cued fear conditioning test. The fear conditioning training protocol employed by Roy et al. (2002) consisted of one training session (2 x 30 s; tone 80 dB; 2 kHz; followed by 2 s shock 0.75 mA) whereas the protocol used by Zhang et al. (2008) consisted of three training sessions (1 x tone 20 s; 80 dB; 2 kHz; followed by 1 s shock 0.70 mA). In the current study the fear conditioning training consisted of one training session (3 x tone 30 s; 70 dB; 5 kHz; followed by 1 s foot shock 0.65 mA DC current). It is possible that the additional training sessions employed by Zhang et al. (2008) facilitated fear association and improved learning compared to the one training session implemented by Roy et al. (2002) and in the current study. Furthermore, in the present study the same animals were tested during adolescence and in adulthood. It is therefore important to consider the potential effects of fear conditioning training during the adolescent period and its potential impact on fear conditioning in adulthood when drawing conclusions with previous studies. In addition, single housing has been shown to impair contextual and cued fear conditioning (Voikar et al., 2005). Given that aggression within male Nr2e1$^{-/-}$ mice necessitated being single housed compared to group housed female *Nr2e1* $^{-/-}$ mice, it is not possible to delineate whether the sex-dependent effects on contextual and cued fear conditioning are due to Tlx deletion or housing conditioning *per se*. Nevertheless, since the amygdala plays a key role in cued fear conditions, these studies suggest that Tlx may also be important in regulating the functions of brain structures beyond the hippocampus, particularly during adolescence.

Deletion of Tlx resulted in a sex-dependent effect on thigmotaxis in the open field. Adolescent and adult male but not female *Nr2e1*$^{-/-}$ mice exhibited a significant reduction in thigmotaxis thus suggesting reduced anxiety-like behaviour. In support, previous studies have reported that adult male *Nr2e1*$^{-/-}$ mice with a targeted disruption of the Tlx locus are less anxious within the elevated plus maze (Roy et al., 2002). It has also been previously shown that adult male *Nr2e1*$^{-/-}$ mice (with a spontaneous deletion of Tlx) display an anxiolytic phenotype independent of sex, but dependent on strain within the elevated plus maze (Young et al., 2002). Indeed, adult male and female *Nr2e1*$^{-/-}$ mice on a C57BL/6J background exhibited reduced anxiety-like behaviour in the elevated plus maze, while *Nr2e1*$^{-/-}$ mice on a B6129F1 background showed similar exploration to control mice (Young et al., 2002). It is important to note that in the latter study, wild type and heterozygous animals were grouped and constituted the control group, while in the present study differences in cued fear memory were observed in adult male *Nr2e1*$^{+/-}$ mice compared to wild type mice. Thus, when data from *Nr2e1*$^{+/-}$ mice is pooled with that from wild type mice, subtle changes in the limbic system circuitry of *Nr2e1*$^{+/-}$ mice may not be picked up. The amygdala plays a key role in both anxiety behaviour and cued fear conditioning (Davidson, 2002; Maren, 2001). Thus, together with the findings in cued fear conditioning, the reduction in thigmotaxis further supports the hypothesis that Tlx can regulate neurobiological processing in brain areas beyond the hippocampus.

Hyperactivity was observed in both male and female *Nr2e1*$^{-/-}$ mice during adolescence and adulthood. This is in agreement with previous studies using the same strain, where

hyperactivity was reported as early as P18 as well as in adulthood (Wong et al., 2010; Young et al., 2002). The findings presented here, in conjunction with previous reports suggest that in the absence of Tlx, the resulting neuroanatomical disruption causes a sex-independent hyperactivity that occurs in adolescence and persists into adulthood. *Nr2e1*$^{-/-}$ mice exhibited a progressive decline in motor performance on the accelerating rotarod at the onset of adulthood. This novel finding suggests that deletion of Tlx causes disruption of cortico-cerebellar/striatal cognitive processing. However, this disruption does not manifest as behavioural impairment until the onset of adulthood, suggesting that Tlx involvement is age-dependent. The impairment in motor performance on the rotarod does not seem to be related to the hyperactive phenotype as both adolescent male and female *Nr2e1*$^{-/-}$ mice are hyperactive, yet impairments in rotarod performance only emerge towards the onset of adulthood.

Previous studies using Nr2e1$^{-/-}$ mice with a spontaneous deletion have reported that mice are physically smaller throughout development and adulthood (Wong et al., 2010; Young et al., 2002). Similarly, we report that both male and female Nr2e1$^{-/-}$ mice exhibit reduced body weight. In addition, we also report that despite smaller body weights, Nr2e1$^{-/-}$ mice exhibit an enhanced growth rate during adolescence. Reduced body weight has also been observed in transgenic mice with a targeted disruption of Tlx, where deviation in postnatal weight gain appears at a similar time point (~P23) to that reported here and previously (Monaghan et al., 1997; Wong et al., 2010). Specifically, Young et al. (2002) have previously reported the body weight of male and female *Nr2e1*$^{-/-}$ mice from embryonic day 12.5 through to adulthood (P70)

and show that a deviation in body weight between wildtype and *Nr2e1*$^{-/-}$ mice occurs at approximately P21. However, when a conditional deletion is implemented in adulthood, body weight is not affected (Zhang et al., 2008). Interestingly, the point of deviation in growth (~P21) coincides with the initiation of hyperactivity (~P18) (Wong et al., 2010). Wong et al. 2010 suggested failure to gain weight at a similar rate to control littermates may be due to the hyperactive phenotype of these mice as they observed no difference in milk consumption of pre-wean *Nr2e1*$^{-/-}$ mice (P0, P7 and P18). This suggests failure to gain weight at a similar rate was not due to a difference in food consumption (Wong et al., 2010). Although in the present study *Nr2e1*$^{-/-}$ mice exhibit a greater growth rate than wild type and *Nr2e1*$^{+/-}$ littermates (Wong et al., 2010). It is likely that hyperactivity stems from underlying neuroanatomical abnormalities resulting from germline deletion of Tlx. However, food intake and metabolism studies have yet to be conducted in adulthood which may help delineate the effect of Tlx deletion on body weight gain.

Sensorimotor observations of wild type, *Nr2e1*$^{+/-}$ and *Nr2e1*$^{-/-}$ mice have been previously reported in early postnatal life and adulthood (Young et al., 2002). However sensorimotor performance during the adolescent period had yet to been fully described and any sex-dependent effect had yet to be characterized. Here, we show that both male and female adolescent *Nr2e1*$^{-/-}$ mice exhibit increased provoked biting (an indication of aggression) which is a well-documented phenotype of this strain (Young et al., 2002). However, while biting was increased in both male and female *Nr2e1*$^{-/-}$ mice in adulthood, it did not reach statistical significance (male, $p = 0.14$ and

female, p = 0.13). Nevertheless, previous studies have reported high aggression in adult male and female *Nr2e1*$^{-/-}$ mice (Abrahams et al., 2005; Young et al., 2002). Defective limbic system functionality in *Nr2e1*$^{-/-}$ mice is thought to play a role in the aggressive phenotype (Monaghan et al., 1997; Young et al., 2002). We also observed impaired eye reflex in male adolescent *Nr2e1*$^{-/-}$ mice. Mice lacking Tlx display hypoplasia of the retina resulting in impaired vision (Miyawaki et al., 2004; Young et al., 2002). While previous studies have shown that this impairment is independent of sex, here female *Nr2e1*$^{-/-}$ mice showed a similar response to wild types. It is unclear why this impairment was observed in a sex-dependent manner. Finally, male adult *Nr2e1*$^{-/-}$ mice display impaired piloerection and palpebral closure, while adult female *Nr2e1*$^{-/-}$ mice show similar primary sensorimotor observations compared to wild type and *Nr2e1*$^{+/-}$ littermates. Together, it seems that the sensorimotor impairments (provoked biting, eye reflex, piloerection and palpebral closure) resulting from the Tlx deletion are somewhat dependent on sex and age.

A potential limitation to the behavioural studies within this strain of *Nr2e1*$^{-/-}$ mice is the potential confound of visual impairment (Corso-Diaz and Simpson, 2015; Young et al., 2002). Thus, it might be suggested that the anxiolytic phenotype observed within the open field in male *Nr2e1*$^{-/-}$ mice could reflect vision impairments. Specifically, mice with reduced vision may unintentionally explore the centre of the arena because they are unaware it is an exposed area of the maze. However, impaired vision has been shown in both sexes (Young et al., 2002). Therefore, a lack of a similar anxiolytic phenotype in female mice suggests that this behavioural phenotype is a

result of neural abnormalities other than visual abnormalities. Moreover, spontaneous alternation and rotarod performance has previously been shown to be unaffected by visual performance (Dember and Roberts, 1958; Morgan et al., 2008) and thus unlikely to be affected by visual impairments in the present study. A second limitation of this study stems from the requirement to single house male knockout mice due to their aggressive phenotype (Young et al., 2002). However, previous studies have shown that spontaneous alternation and motor performance on the rotarod are unaffected by housing conditions in C57BL/6 mice (Voikar et al., 2005). Furthermore, previous studies have shown that singly housed *Nr2e1*$^{-/-}$ mice exhibit reduced body weight and increased hyperactivity compared to single housed wild type littermates (Voikar et al., 2005). Thus, suggesting that social isolation does not account for the reduced body weight and hyperactivity of *Nr2e1*$^{-/-}$ mice observed within this study. Notwithstanding that social isolation may impact upon fear conditioning, overall the evidence suggests that the impairments in motor, cognitive and anxiety-related behaviours assessed here are likely a function of Tlx deletion rather than housing conditions.

Given the well-established role of Tlx as a transcriptional repressor of downstream target genes, it is important to consider the molecular mechanisms which may underpin the discrepancies in behaviour between wildtype and Tlx-deficient mice in the current study and indeed the developmental time points at which these changes emerge. Tlx has been shown to recruit the epigenetic modulators lysine-specific histone demethylase 1 (LSD1) and histone deacetylases (HDAC) 3 and 5 to regulate gene expression (Sun et al., 2010; Sun et al., 2007). In turn, expression of an array of

genes has been shown to be regulated by Tlx and of particular interest are p21 and Pten as they are involved in adult hippocampal neurogenesis (Amiri et al., 2012; Pechnick et al., 2008). Indeed Pten has been shown to have a role in hippocampal-dependent contextual fear conditioning in mice (Lugo et al., 2013). Because adolescence is a significant developmental period for the remodelling of hippocampal connectivity and networking including neurogenesis, Tlx-regulated genes such as p21 and pten may have important roles to play in mediating the associated behavioural changes at this time. Future studies will help elucidate this theory.

A number of studies have shown that deletion of Tlx causes neuroanatomical abnormalities similar to those observed in bipolar disorder and schizophrenia, such as enlarged ventricles and reduced volume of the hippocampus, cerebral cortex and amygdala, as well as impaired neurogenesis (Andreazza and Young, 2014; Ross et al., 2006; Strakowski et al., 2012). Moreover, genetic variation at the *Nr2e1* locus in humans has been linked to susceptibility of developing bipolar disorder (Kumar et al., 2008). Furthermore, the behavioural abnormalities of *Nr2e1* mice are similar to those observed in bipolar disorder i.e., aggression, hyperactivity and impaired learning (Ballester et al., 2012; Latalova, 2009; Najt et al., 2007; Vohringer et al., 2013). Interestingly, these disorders manifest primarily during the adolescent period, and this aligns with the behavioural observations in the adolescent Tlx deficient mice in the current study (Giedd et al., 2008). Thus, the observed impairment in limbic system structure and function may indicate a potential role of Tlx in mood disorders. In conclusion, we show that deletion of Tlx results in impairment in motor, cognitive and

anxiety-related behaviours during adolescence and adulthood in both male and female mice with the majority of effects occurring during adolescence rather than adulthood. We also show that there is a progressive decline in motor performance of *Nr2e1*$^{-/-}$ mice in adulthood thus indicating cortico-cerebellar/striatal dysfunction in these mice. This novel finding together with alterations in amygdala-dependent behaviour suggests a function for Tlx beyond its regulation of adult neurogenesis in the hippocampus. Adolescence is a critical period for postnatal brain maturation and thus susceptibility to emotional and cognitive-related disorders. Given that the role of Tlx in the regulation of cognitive and anxiety-related behaviour is most apparent during adolescence, Tlx is poised to be a key target in understanding the emergence of neurobiological disorders at the onset of adolescence and early adulthood.

Chapter 6

TLX knockdown in the dorsal dentate gyrus of juvenile rats differentially affects adolescent and adult behaviour

Danka A. Kozareva[a,b], Tara Foley[a], Gerard M. Moloney[a], John F. Cryan[a,b], Yvonne M, Nolan[a,b]

[a] Department of Anatomy & Neuroscience, University College Cork, Ireland

[b] APC Microbiome Institute, University College Cork, Ireland

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Abstract

The orphan nuclear receptor TLX is predominantly expressed in the central nervous system and is an important factor regulating the maintenance and self-renewal of neural stem cells from embryonic development through adulthood. In adolescence and adulthood, TLX expression is restricted to the neurogenic niches of the brain: the dentate gyrus of the hippocampus and the subventricular zone. The adolescent period is critical for maturation of the hippocampus with heightened levels of neurogenesis observed in rodents. Therefore, we investigated whether lentiviral silencing of TLX expression (TLX knockdown) in the dorsal dentate gyrus in juvenile rats incurred differential impairments in behaviour during late adolescence and adulthood. Our results showed that knockdown of TLX in the dorsal dentate gyrus led to a decrease in cell proliferation in the dorsal but not ventral dentate gyrus. At a behavioural level we observed differential effects in adolescence and adulthood across a number of parameters. A hyperactive phenotype was present in adolescent but not adult TLX knockdown rats, and an increase in immobility during adolescence and in swimming frequency during adulthood was observed in the forced swim test. There was an increased defecation frequency in the open field during adulthood but not adolescence. There were no changes in cognitive performance on hippocampus-dependent tasks or in anxiety-related behaviours. In conclusion, silencing of TLX in the dorsal dentate gyrus led to impairments in hippocampal-independent behaviours which either did not persist or were reversed during adulthood. The current data highlight the temporal importance and function of the nuclear receptor TLX during development.

Key words: Adolescence; dentate gyrus; TLX; hyperactivity; anxiety; cognition

6.1 Introduction

Adolescence is a critical period during which significant changes in neuroendocrine, neurodevelopmental and behavioural systems take place (McCormick and Mathews, 2010; Spear, 2000b)C(Romer, 2010). During this period, neurodevelopmental circuits related to learning and memory, emotional regulation and decision making undergo maturation across the hippocampus (Murty et al., 2016), amygdala (McCormick and Mathews, 2010) and prefrontal cortex (Murty et al., 2016; Yurgelun-Todd, 2007), thereby rendering changes in cognition related to executive function and cognitive control (reviewed by (Hueston et al., 2017)). In rodents, adolescence is between postnatal day (P) 21 to P 60; up until P42 is generally considered to be the juvenile period, and P42 to P60 is considered adolescence (Hueston et al., 2017; McCutcheon and Marinelli, 2009; Spear, 2000a). Within the rodent hippocampus specifically, adolescent development presents with an increase in the number of granule cells and in the volume of hippocampal layers (Bayer, 1982; Hueston et al., 2017; McCutcheon and Marinelli, 2009; Spear, 2000a). It has been demonstrated in both the human and rodent hippocampus that the number of newborn neurons is up to four times higher during adolescence than the number of neurons born during adulthood (Curlik et al., 2014; Kuhn et al., 1996; Spalding et al., 2013). This process of neurogenesis (the generation of new neurons from neural stem/progenitor cells (NS/PCs)) occurs throughout the life span of most mammalian species in discrete neurogenic niches of the central nervous system (van Praag et al., 2002). The subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus is one such neurogenic niche, where newborn granule cells become integrated in the local neuronal network (Vicario-Abejon et al., 2000). Ablating and/or decreasing adult hippocampal neurogenesis has

been shown to result in specific deficits such as impairment in long-term memory retention in the Morris Water Maze test in rats (MWM; (Snyder et al., 2005)), decline in performance on contextual fear conditioning tasks in mice (Saxe et al., 2006), impairment in pattern separation in mice (Clelland et al., 2009; Nakashiba et al., 2012; Sahay et al., 2011) and increased behavioural despair in the forced swim test in mice (FST; (Snyder et al., 2011)). Ablating hippocampal neurogenesis during adolescence in rats has been shown to result in a decrease in cell proliferation and neuronal survival during adulthood, as well as impaired performance in MWM task and fear conditioning (Achanta et al., 2009). Human studies have also shown that brain irradiation used for cancer treatment during adolescence was associated with long-term decreases in IQ scores and other behavioural changes (Rodgers et al., 2013). Additionally, disorders commonly arising during adolescence such as schizophrenia, drug abuse, attention deficit hyperactivity disorder and depression have been associated with changes in hippocampal neurogenesis (reviewed by (Hueston et al., 2017)). Currently our understanding of the relative contribution of hippocampal neurogenesis during adolescence to cognitive function and regulation of emotions such as anxiety- and stress-related behaviours and/or disease development during adulthood is limited.

Recent attention has focused on the role of nuclear receptors in neurogenesis. Nuclear receptors are a superfamily of transcription factors that regulate genes involved in physiological and developmental processes and have proven to be important drug targets for a host of diseases (Sladek, 2003). The orphan nuclear receptor subfamily 2

group E member 1 (Nr2e1), commonly known as TLX, is an evolutionary conserved member of the nuclear receptor superfamily found in both vertebrates and invertebrates (Mangelsdorf et al., 1995). An alignment of drosophila, murine and human TLX proteins reveals remarkable interspecies conservation with 70% - 99% homology between the three species (Yu et al., 1994). Expression of TLX is specific to the developing forebrain and retina. In the mouse embryo, TLX expression is detectable at embryonic day 8 (E8), peaks at E14 and declines thereafter. Postnatal TLX expression increases with high levels present in the neurogenic niches of the adult brain and more specifically in the NPCs in the SGZ and subventricular zone (Li et al., 2008b; Monaghan et al., 1995). The functional importance of TLX is now apparent from studies showing that TLX maintains adult hippocampal NPCs in a proliferative, undifferentiated state (Shi et al., 2004; Sun et al., 2007). It has also been reported that TLX controls the activation status and the proliferative ability of hippocampal NPCs by repressing cell cycle-related genes such as *pten* (Niu et al., 2011). Findings from our lab and others suggest the involvement of TLX in aggression-, cognition- and anxiety-related behaviours as well as in motor performance during adolescence and adulthood (Christie et al., 2006; O'Leary et al., 2016a; O'Leary et al., 2016b; Zhang et al., 2008). We have also shown that deletion of TLX impairs exercise-induced hippocampal neurogenesis during adolescence (Kozareva et al., 2017b). Nonetheless, some discrepancies across these studies have emerged due to the time during the lifespan and the location at which the TLX knockdown occurred (spontaneous deletion from birth versus targeted deletion during adulthood, for review see (O'Leary et al., 2016b). It has been suggested that an important confounding factor for the spontaneous deletion and homologous recombination deletion models is the fact that TLX signaling

has been disrupted from early life when important developmental processes take place. Additionally, we have shown that spontaneous deletion of TLX from embryo results in different behavioural outcomes during adolescence and adulthood, respectively, with greater impairments during the adolescent period (O'Leary et al., 2016a). Interestingly, distinct hippocampal functions have been associated with the separate anatomical regions of the rodent hippocampus in septo-temporal order (O'Leary and Cryan, 2014). For instance, the dorsal hippocampus has been shown to be primarily involved in spatial learning and memory, while a predominant role of the ventral hippocampus has been displayed in the stress response and anxiety-related behaviour (Bannerman et al., 2004; Fanselow and Dong, 2010). Furthermore, functional segregation of hippocampal neurogenesis along the septo-temporal axis has also been implicated. Evidence from rodent studies of behavioural, pharmacological and genetic models of depression suggests that each of these interventions elicit differential regional effects on specific stages of the neurogenic process (O'Leary and Cryan, 2014; Tanti and Belzung, 2013). This could further account for the discrepancies in findings from TLX deletion studies when looking at neurogenesis-associated behaviours without functionally disseminating them across the dorso-ventral axis. The aim of the present study was thus to investigate the effect of TLX knockdown using a lentiviral approach to target NPCs in the rat dorsal DG (dDG) during early adolescence on behaviours in late adolescence and adulthood.

6.2 Materials and Methods

6.2.1 Experimental design

Behavioural analysis was carried out in two cohorts of male rats - adult and adolescent, who had undergone either a targeted silencing of the *Nr2e1* gene (TLX) or GFP-tagged scrambled RNA administration as control in the dDG at P28. Behavioural testing commenced at P42 and P90 for the adolescent and adult cohorts, respectively. Open field test, a modified version of spontaneous location recognition task, elevated plus maze (EPM), cued and contextual fear conditioning and FST were conducted in both cohorts. Only the adult group underwent testing in spontaneous and discrete alternation in the Y maze (see Figure 6.1 for experimental design).

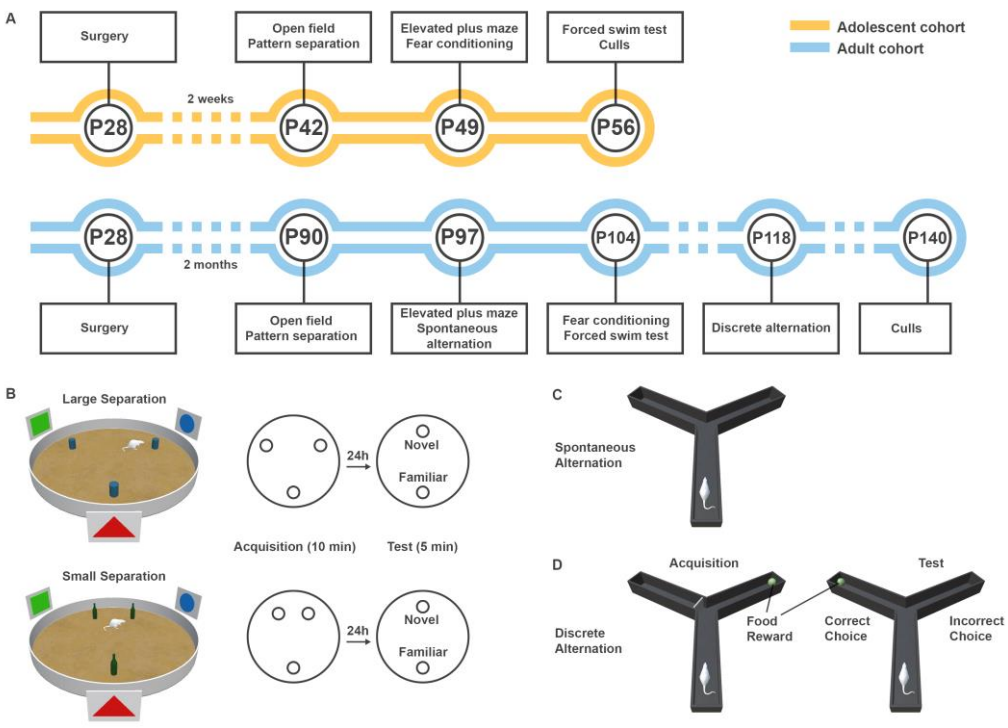


Figure 6.1: Experimental design and apparatus
Outline of the experimental groups and timeline illustrating the duration of the experiment (A). Each circle illustrates the day in which a certain set of behavioural testing has begun. The tests were

performed over a number of days (the number of days depends on the behavioural protocol for each test). Drawing depicting the apparatus used for the pattern separation task including the proximal spatial cues (B). Figure adapted from (Bekinschtein et al., 2014). Drawing depicting the Y-maze apparatus with its three identical arms equidistant at 120° angle from each other (C). Schematic representation of the discrete alternation task in the Y-maze (D).

6.2.2 Animals

Male Sprague-Dawley rats (n=10-12) were bred in house, weaned at P21 and group housed on a 12:12-h light-dark cycle in a temperature-controlled colony room (22-24°C). Food and water were available at all times except when the adult cohort underwent a food restriction protocol for 5 days, during which time they were maintained at 90% of free feeding weight in preparation for the discrete alternation in the Y maze training. All procedures were conducted in accordance with the European Directive 2010/63/EU, and under an authorization issued by the Health Products Regulatory Authority Ireland and approved by the animal Ethics Committee of University College Cork.

6.2.3 Stereotaxic gene delivery

One week post weaning (P28), rats were anaesthetized with isoflurane/O_2 (2.5 – 4.5%), and bilaterally stereotaxically infused with 2.0µl FIV-based short-hairpin RNA vectors (shRNA targeting mouse TLX >$1x10^7$ IU mL^{-1} or scrambled control >$1x10^7$ IU mL^{-1}) into the dDG (from bregma: AP: -3.3mm; ML: ±2mm; DV: -3.0mm) at 1µl/min. Lentiviral particles pseudotyped with the vesicular stomatitis virus (VSV-G) glycoprotein were purchased from Genecopoeia (shRNA Cat# LP-MSH039783-LvU6-0205-m; scrambled control Cat# LP-CSHCTR001-LvU6-0205). The shRNA contained 4 expression constructs designed to silence TLX expression via RNA interference driven by U6 promoter with guaranteed knockdown effect of 70% or more

($LV^{TLXShRNA}$), while the scrambled control contained the same nucleotide sequence, but randomly rearranged, thus non-silencing (LV^{SCR}), and driven by the same promoter (Genecopoeia, Rockville, MD, USA). Both vectors carried *eGFP* reporter gene clone driven by SV40 and IRES promoters.

6.2.4 Behavioural tests

6.2.4.1 Open field test

Spontaneous exploratory locomotor activity and thigmotaxis in the open field were used as a general measure of motor function and anxiety-related behaviours, respectively (Cryan and Sweeney, 2011). At the beginning of each trial, animals were placed in the centre of a circular white open field arena with diameter 90cm and diameter of the centre area 45 cm (made in house) for 10 min. The arena was cleaned with 70% ethanol to avoid cue smell between each trial. The room was brightly lit, which in combination with the exposure to novel environment and open space, induce anxiety in rodents. Thus, anxiogenesis was measured by time spent in centre vs. time spent in the edges of the open field arena. Locomotor activity was measured by distance travelled and average speed over the 10-minute-exploration, where large distance travelled and increased velocity are indicative of hyperactivity (Markel et al., 1989). Both thigmotaxis and locomotor activity were measured and analysed using a specialized software with tracking system (Ethovision XT 8.5, Noldus Information Technology, USA).

6.2.4.2 Pattern separation test

In order to measure the ability of rats to discriminate similar memory representations (a neurogenesis-associated task; Sahay et al., 2011; Sahay et al., 2011a; Gandy et al., 2017) we adopted the modified spontaneous location recognition paradigm, which tests pattern separation (Bekinschtein et al., 2014). The floor of an open field arena was covered with standard cage bedding, and the light in the room was dimmed. The arena was placed in the middle of the room and was surrounded by three proximal spatial cues and distal standard furniture. Each rat was habituated to the arena and the room for 10 min for 5 consecutive days before the start of the testing. On day 6, the acquisition phase took place, where rats were placed in the arena with 3 identical objects, fixed to the floor of the arena and placed along the circumference 15 cm away from the wall and 30 cm away from the centre of the arena. The objects were either cans or bottles with labels removed and were cleaned with 50% ethanol between trials. During acquisition, the three objects were either placed equidistant (120°, 49 cm apart) for the large separation task, or two of the objects were placed close together (50°, 20.5 cm apart) and the third object at an equal distance from the other two, for the small separation task. Rats were left to explore the arena with the objects for 10 min and 24 hours later the test phase took place. During the test, two objects (identical to the objects used during the acquisition phase) were positioned diametrically opposite to each other. One of the objects was in familiar location, while one was in a novel location. The novel location was chosen such that the object was positioned exactly in between the two familiar locations of the acquisition phase (Figure 6.1; 180° from the familiar object). Each animal was tested twice in counterbalanced order for the large and small separation. Scores are presented as discrimination ratio, which is calculated

by subtracting time spent with familiar object from time spent with novel object and dividing the result by total exploration time (Bekinschtein et al., 2014).

6.2.4.3 Elevated plus maze test

The EPM test was is a widely used test of approach-avoidance-related anxiety(Cryan and Sweeney, 2011). The apparatus consists of two open (50.5 x 10.5 cm) and two enclosed (50.5 x 10.5 x 40 cm) arms, which extend from and are connected by a common central square (10.5 x 10.5 cm). The maze was elevated above the floor on a central pedestal (75 cm; Cat# ENV-560; Med Associates Inc). The open arms of the maze are considered to be more aversive for rodents than the closed ones and anxiolytic behaviour is defined as increased number of entries and time spent in the open arms (Cryan and Sweeney, 2011). Animal behaviour was videotaped for the duration of a 5-minute test and the number of entries in closed and open arms was analysed along with the percent time (of total test time) spent in either closed or open arms, respectively. An arm entry was defined as the front two paws of the animal being within the borders of the arm (two-paw criterion).

6.2.4.4 Y maze test

The two Y-maze tests described below were performed only on the adult cohort. The experimental apparatus, made in house, was a black Y-maze made of wood. Its arms (50 cm x 10 cm x 30 cm) were connected to form a triangular space in the centre (10 cm x 10 cm x 10 cm), and not belonging to either arm **(**Figure 6.1**)**. The same apparatus was used for both tests.

6.2.4.4.1. Spontaneous alternation in the Y maze

The spontaneous alternation in the Y-maze test was utilized as a measure spatial working memory. This behavioural test was validated previously in mice (Senechal et al., 2007). Briefly, each animal underwent one trial and the Y-maze was cleaned with 70% ethanol between animals. The rat was placed into the outward-extending end of one arm (always the same) facing the wall of the arm and was then allowed free exploration of the maze for 5 min. Arms were numbered (1-3) and the sequence of arm entries was recorded manually during the test, where an arm entry was defined by the four-paw criterion (Senechal et al., 2007). A spontaneous alternation is defined as the consecutive entry in all three arms. The percentage spontaneous alternations, i.e. the ratio of actual to maximum number of alternations completed by the animal within 5 minutes was calculated (Senechal et al., 2007).

6.2.4.4.2. Discrete alternation in the Y maze

The discrete alternation in the Y-maze test is sensitive to dysfunction and/or lesions of the hippocampus as well as to subtle manipulations of hippocampal synaptic plasticity (McHugh et al., 2008). In the discrete-reward alternation test, successful alternations were rewarded with sugar pellets; the test was performed as documented and validated previously (Deacon and Rawlins, 2006). Briefly, 3 days prior to habituation and testing, rats were placed on a food-restricted diet and reduced to ~90% of their free-feeding weight. During habituation, cage-mate paired rats were placed into the start arm (always the same) of the Y-maze and left to explore freely and collect food rewards. Two food pellets were placed at either end of the two arms that animals were tested in. Rats were then left to explore until they ate the pellets or for 3 min.

This was repeated 4 times a day with at least a ten-minute inter-trial interval. During the acquisition day, or forced-trial training (Pioli et al., 2014), each rat was placed in the start arm and forced to visit a baited arm by blocking the access to the other arm (Figure 6.1). The order of left-right forced choices was determined by a pseudorandom sequence. Each trial lasted 1 min or until the rat ate the food pellets and each rat underwent 10 forced trials. During testing, animals received ten trials a day for two consecutive days. The inter-trial interval was 10 min. Each test trial consisted of two runs – a forced run and a free run. The forced run was performed identical to the acquisition day. Ten (test day 1) or sixty (test day 2) seconds after the end of the forced run, the free run was performed. At the beginning of the free run, the rat was returned to the start arm and allowed to choose either goal arm. If the animals chose the arm opposite the one they had been forced into during the forced run, they received food pellets. If the same arm that they had been forced into was chosen, they received no food reward. The percentage correct trials performed by each animal was calculated (Bardgett et al., 2008; Deacon and Rawlins, 2006).

6.2.4.5 Fear conditioning: contextual and cued

Hippocampal-dependent learning (contextual) and amygdala-dependent processing (cued) were examined using the fear conditioning paradigm as previously described (Maren, 2001; Pattwell et al., 2011). On day 1 (acquisition), rats were placed into the fear conditioning chambers (Med. Associates, USA: 30.5 cm x 24.1 cm x 21 cm) which were fragranced with lemon and ginger from a tea bag placed under the grids of the flooring. Animals were allowed to explore the chamber for two minutes during the habituation period, followed by the delivery of three foot shocks paired with tones

(30 s tone: 5 kHz; 70 dB; 1 s foot shock: 0.60 mA DC current) separated by 30-second intervals. One minute after the last shock, animals were removed from the chambers and placed back in their cages. On day 2, contextual fear memory was measured by placing the rats back in the same chambers for 5 min and 30 s, but in the absence of tone-shock pairing. On day 3, cued fear conditioning was assessed by placing the animals in the same chambers but changing the context to a novel one (white solid floor; black wall insert at 60°; almond scent). Two minutes after the start of the test, three tones were presented without being paired with shock (30 s: 5 kHz; 70 dB). Behaviour was recorded and the percentage time spent in freeze position was calculated with specialized software (Video freeze, Med Associates, USA) for the duration of the whole test or during the 30-second tone presentations for the context and cued fear conditioning test, respectively.

6.2.4.6 Forced swim test

The FST was performed as previously described (Slattery and Cryan, 2012). In brief, rats were placed individually in Pyrex cylinders (Fischer Scientific: 21 cm x 46 cm) for 15 min. Cylinders contained water at 25°C up to a 30 cm mark on the cylinder. Behaviours were recorded by video camera for subsequent analysis. The 15-min session was divided into three separate triads and was analysed as previously described (Cryan et al., 2005). For this purpose, a time-sampling technique was employed, whereby the predominant behaviour (immobile, swimming, or climbing) in each 5-second period of the 5-minute interval was recorded. When the rat performed the most minimal movements (or none at all) to keep its head afloat, the behaviour was scored as immobile. When the rat was moving horizontally through the chamber crossing

quadrants, swimming behaviour was assigned. Finally, climbing was defined as upward-directed movements of the forepaws along the side of the swim chamber. Scores are presented as cumulative for each triad and for the full 15-minute duration of the test.

6.2.5 Tissue preparation

At P56, rats from the adolescent cohort were euthanized with an intraperitoneal injection of anaesthetic (Pentobarbital: 50 mg/kg) and transcardially perfused using a 0.1M phosphate buffered saline (PBS) solution followed by 4.0% paraformaldehyde (PFA) in PBS. After overnight incubation in PFA, brains were incubated in 30% sucrose until they sank, and subsequently flash frozen using liquid nitrogen. At P140 rats from the adult cohort were sacrificed by rapid decapitation (n=8 of each group). Freshly dissected hippocampal tissue from the adult rats was processed for molecular analysis by qRT-PCR as described below. In order to validate virus expression was sustained into adulthood, n=2 of each group were perfused and the brains were processed as described above.

6.2.6 Immunohistochemistry

Coronal sections (40 μm) through the hippocampus from all perfused brains were collected onto slides in a 1:12 series, then stored at -80°C. To confirm virus spread and silencing of TLX, sections were assessed for GFP and TLX immunoreactivity, respectively. Sections were washed, incubated in 3% normal donkey serum (NDS: Sigma D9663) and then in anti-GFP raised in rabbit (GeneTex; GTX113617; 1:250) or anti-TLX raised in rabbit (Abcam ab86276; 1:100) antibodies overnight at 4°C.

Following that, sections were washed and incubated in AlexaFluor488 donkey anti-rabbit (ab150073; 1:500) antibody for two hours at room temperature. Subsequently, sections were washed, counterstained with DAPI (Sigma D9642; 1:5000) and coverslipped using anti-fade mounting medium (Dako; S3023). To assess cell proliferation, immunohistochemistry (IHC) for the Ki-67 antigen was performed. Briefly, sections were incubated in H_2O_2 (in 1% methanol, 40 min at room temperature) and then blocked in 10% normal goat serum (NGS; Sigma; G9023). Sections were incubated with rabbit anti-Ki67 (GeneTex; GTX16667; 1:250; overnight at 4°C), followed by sequential incubations with the streptavidin-biotin immunoenzymatic antigen detection system (Abcam; ab64261) and coverslipped using DPX mounting medium (Sigma; 44581).

6.2.7 Image analysis and cell quantification

Fluorescent images were obtained with Olympus FV1000 scanning laser confocal system (BioSciences Imaging Centre, Department of Anatomy and Neuroscience, University College Cork, Ireland), incorporating an IX81 inverted microscope. Z-stack images with 4.4 μm step size were collected using a 10X objective, which has a NA of 0.40 and is a UPlanSApo objective, highly corrected for both spherical and colour aberration. Excitation light source used was a laser diode for 405 nm and a multiline argon laser for 488 nm. The filter set used was an excitation dichroic mirror set DM 405/488/543. Emission light was detected using a carriable barrier filter, which for DAPI and Alexa Fluor 488 was set to 425-475 nm and 500-530 nm, respectively. Emission light is detected with three photomultiplier detectors. The software used for image acquisition was Olympus Fluoview Ver4.2b. For bright field images, an

Olympus BX533 upright microscope coupled to an Olympus DP72 camera was used with a 10X objective. The DG was imaged bilaterally on all sections.

In order to quantify TLX staining through the whole DG, mean fluorescence intensity was measured across a randomly selected area within the DG and mean background fluorescence intensity was measured across a randomly selected area of the same size outside the DG. TLX staining was then expressed as the ratio between fluorescence within to fluorescence outside of the DG. For cell quantification, systematic random sampling was used through the whole DG by counting the cells on both hemispheres in 1:12 series (480 μm apart). Analysis of both fluorescence intensity and cell number quantifications was performed using the image software package ImageJ (National Institute of Health, USA). Cell numbers and fluorescence intensity ratio were expressed as an average per section in both the dDG and vDG according to the coordinates: dDG as -2.8 to – 4.0 from Bregma; vDG as -4.0 to -6.3 (Ritov et al., 2014).

6.2.8 Quantitative RT-PCR analysis of hippocampal tissue

Samples were processed according to the GenElute kit protocol (Sigma; RTN350). Briefly, total cellular RNA was homogenized into lysis solution and homogenized sample was filtered through a binding column to remove non-RNA from the sample. Equal volume of 70% ethanol was added to the filtrate and purified through columns, which were then washed with buffer. Purified mRNA was recovered into 30 μL of elution solution. Samples were further treated with DNase1 (Sigma; AMPD1) to

ensure the complete removal of endogenous DNA from the samples. Total RNA yield and purity were determined using the Nanodrop System (Thermo Scientific). Synthesis of cDNA was performed using 0.5 μg of normalized total RNA from each sample using ReadyScriipt cDNA synthesis mix (Sigma; RDRT-25RXN). Probed cDNA amplification was performed in a 20 μL reaction consisting of 10 μL KiCqStart qPCR Mix with ROX (Sigma; KCQS02), 0.1 μL of each forward and reverse primer (final concentration 250 nM), 1 μL cDNA template, and 8.8 μL RNase-free water. Real time RT PCR was performed in duplicate in a 96-well plate (Applied Biosystems) and captured in real time using the StepOne Plus System (Applied Biosystems). Relative gene expression was adjusted to the housekeeper *Actb* (Actin Beta), and quantified using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

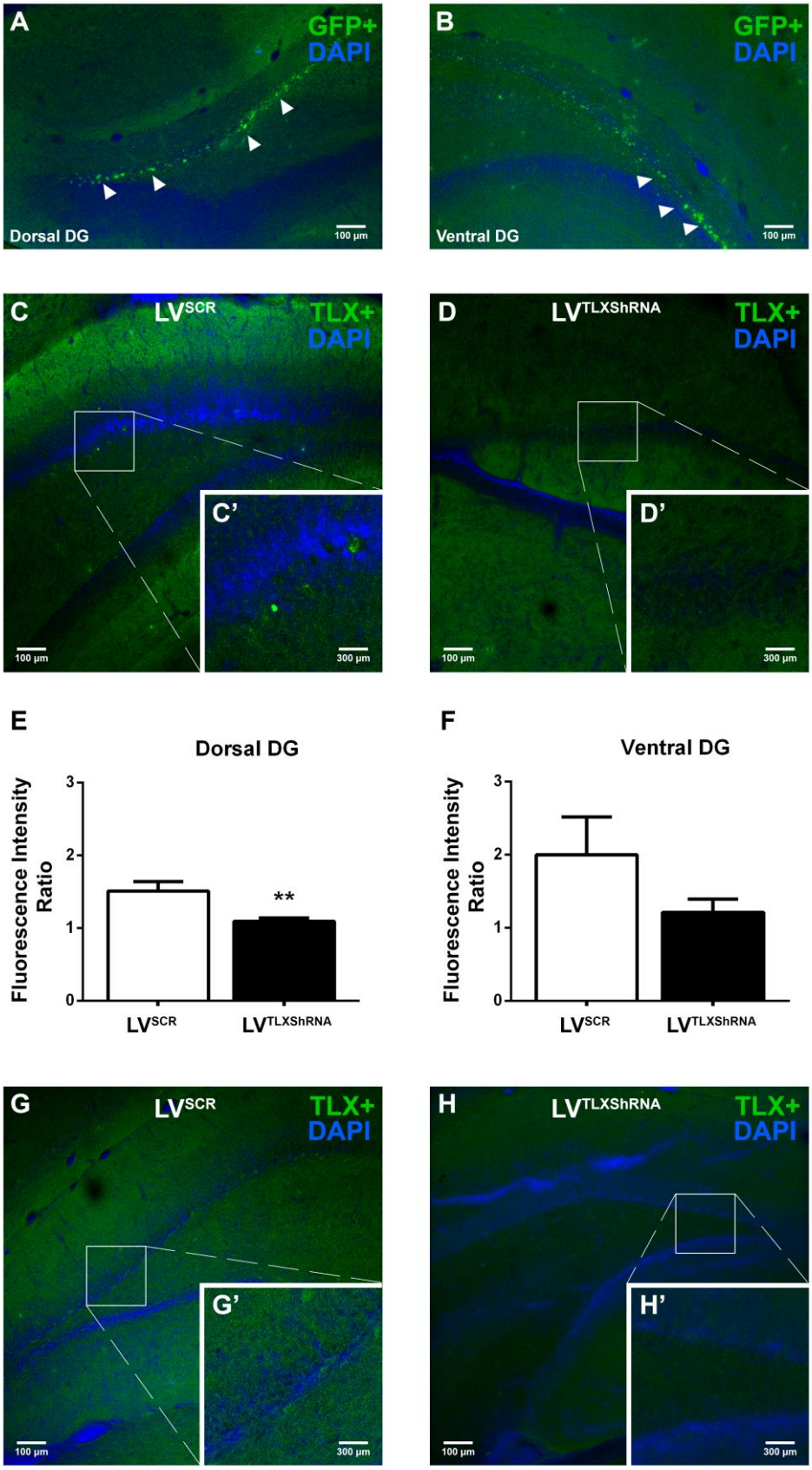
6.2.9 Statistical analysis

All data were analysed using SPSS statistical software (SPSS 17.0, Chicago, IL). Data were analysed by an independent-sample t-test except in the case of cued fear conditioning, when repeated measures ANOVA was employed followed by Bonferoni post hoc analysis. Group means from pattern separation test were compared against chance level by one-sample t-test. An alpha level of 0.05 was used as criterion for statistical significance. All data are presented as mean ± SEM.

6.3 Results

6.3.1 Lentiviral transduction of the rat dDG resulted in TLX knockdown and reduced the number of proliferating cells in the dorsal granule cell layer

GFP was expressed in both the dorsal and ventral hippocampus of all adolescent rats (n=10-12). Representative images show that GFP+ cells were evident in the SGZ of both the dDG (Figure 6.2) and vDG (Figure 6.2) of all animals at four weeks following surgery, demonstrating successful transduction of cells in the neurogenic niche by the lentivirus (Figure 6.2, white arrowheads). Representative images show reduced fluorescence intensity of TLX after probing with TLX antibody by IHC in the DG of rats that underwent TLX knockdown $LV^{TLXShRNA}$ (Figure 6.2) compared to control LV^{SCR} treated (Figure 6.2). TLX expression was significantly decreased in the dDG (t (12) = 3.456, $p < 0.05$; Figure 6.2) but not vDG (Figure 6.2) of TLX knockdown ($LV^{TLXShRNA}$) compared to control (LV^{SCR}) treated rats. Representative images of TLX immunohistochemistry in the adult hippocampus show a decrease in TLX immunostaining in rats injected with TLX knockdown rats ($LV^{TLXShRNA}$ (Figure 6.2)) compared to control (LV^{SCR}) treated rats (Figure 6.2) demonstrating that TLX knockdown was sustained into adulthood.


A
GFP+
DAPI
Dorsal DG
100 µm
B
GFP+
DAPI
Ventral DG
100 µm
C
LVSCR
TLX+
DAPI
C'
100 µm
300 µm
D
LVTLXShRNA
TLX+
DAPI
D'
100 µm
300 µm
E
Dorsal DG
Fluorescence Intensity Ratio
3
2
1
0
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LVSCR
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Ventral DG
Fluorescence Intensity Ratio
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LVSCR
LVTLXShRNA
G
LVSCR
TLX+
DAPI
G'
100 µm
300 µm
H
LVTLXShRNA
TLX+
DAPI
H'
100 µm
300 µm

Figure 6.2: Lentiviral transduction of the rat dDG resulted in TLX knockdown.
Representative confocal images illustrating virus localization after stereotaxic surgery. Immunohistochemichal staining shows GFP+ (green; arrowheads) cells and DAPI+ (blue) nuclei in the dorsal (A) and ventral (B) DG of $LV^{TLXShRNA}$ treated rats. Images were taken at 10X magnification. Scale bar = 100 μm. Representative confocal images of coronal sections through the dDG immunohistochemically stained with TLX (green) and DAPI (blue) from adolescent control (C) and TLX knockdown (D) rats. TLX fluorescence intensity measured as ratio of fluorescence intensity within the DG divided by fluorescence intensity outside of the DG in the dorsal (E) and ventral (F) DG of adolescent rats transduced with either LV^{SCR} (white bars) or $LV^{TLXShRNA}$ (black bars). Data are expressed as mean ± SEM. ** $p < 0.01$; independent-sample t-test, n = 6-8 for dDG and n = 3-7 for vDG. Representative confocal images of coronal sections through the dDG immunohistochemically stained with TLX (green) and DAPI (blue) from adult control (G) and TLX knockdown (H) rats. Images were taken at 10X magnification. Scale bar = 100μm. Insets (C', D', G', H') depict zoomed in image of areas selected (white square) from the respective lower magnification images (C, D, G, H, respectively). Scale bar = 300 μm.

To validate that TLX knockdown induced an impairment in cell proliferation, Ki67-immunopositive cells were counted in adolescent rats that had been injected with either $LV^{TLXShRNA}$ or LV^{SCR}. TLX knockdown ($LV^{TLXShRNA}$ treated) rats had on average 39.28 proliferating cells (SD = 11.37) in the whole of the dDG, while the mean number of Ki67+ cells in the dDG of control (LV^{SCR} treated) adolescent rats was 52.17 (SD = 5.7). Despite this difference not being significant, the effect size was moderate to strong (r = 0.4; Figure 6.3). The number of proliferating cells in the whole vDG was not affected by TLX knockdown ($LV^{TLXShRNA}$ treatment) during adolescence **(**Figure 6.3). There was a significant decrease in Ki67+ cells in the dorsal granular cell layer (GCL (t (6) = 3.203, $p < 0.05$; Figure 6.3) but not the ventral GCL (Figure 6.3) of TLX knockdown ($LV^{TLXShRNA}$ treated) rats compared to the respective dorsal and ventral GCLs of the control (LV^{SCR} treated) group. No differences in the mean numbers of Ki67+ cells were observed in the hilus across the septo-temporal axis (Figure 6.3). The representative image of the DG of LV^{SCR} treated rats shows multiple clusters of Ki67+ cells (Figure 6.3; arrowheads), while fewer numbers of cells can be observed in the DG of TLX knockdown ($LV^{TLXShRNA}$ treated) rats (Figure 6.3; arrowheads). Furthermore, a trend towards a decrease in DCX expression measured by qRT-PCR

was observed in the hippocampi of TLX knockdown compared to control rats (Figure 6.3**)**.

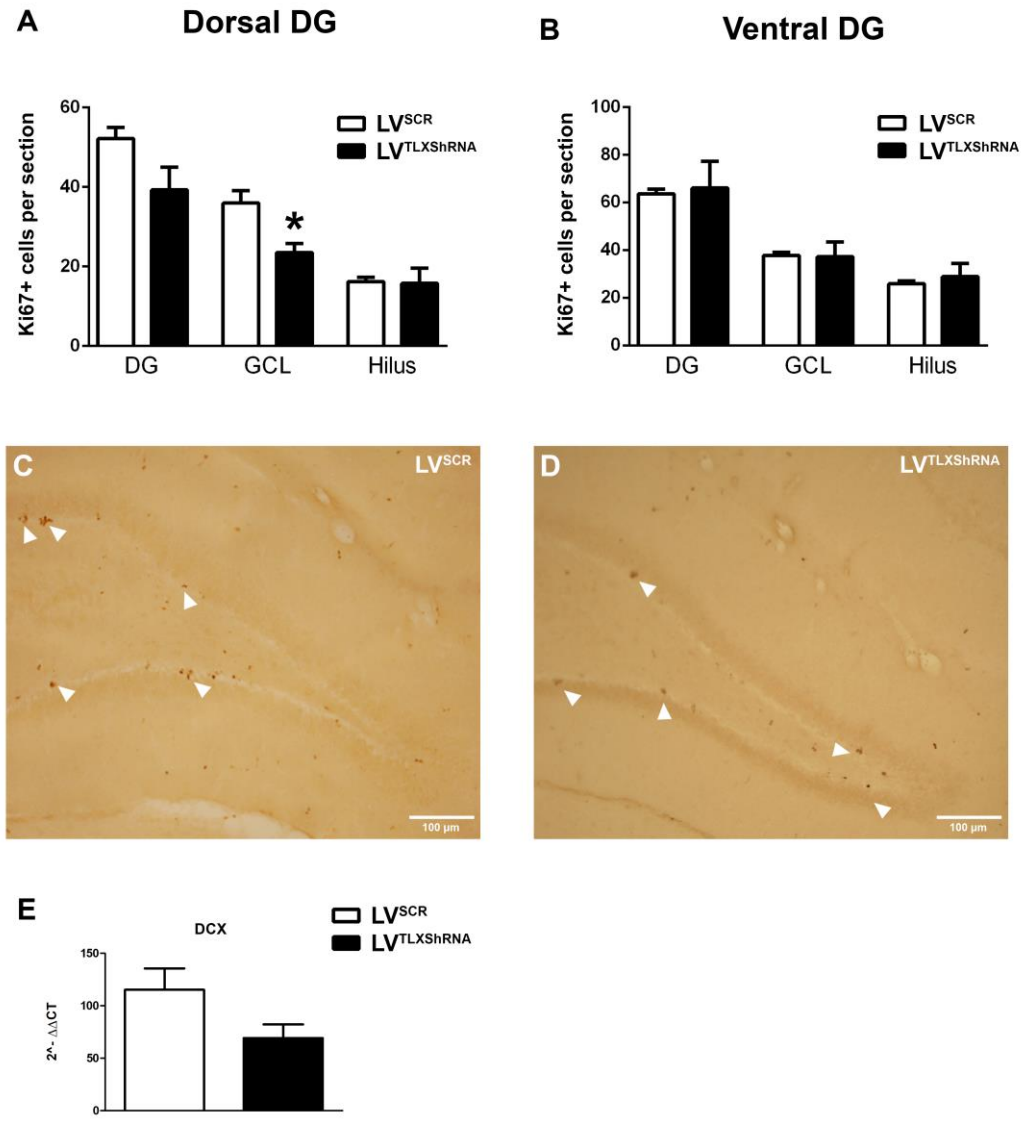


Figure 6.3: Lentiviral transduction of the rat dDG reduced number of proliferating cells in the dDG. Mean number of Ki67+ cells per section in the dorsal (A) and ventral (B) DG of adolescent rats transduced with either LV^{SCR} (white bars) or $LV^{TLXShRNA}$ (black bars). Data are expressed as mean ± SEM. * $p < 0.05$; independent-sample t-test, $n = 4$. Representative bright field images of coronal sections through the DG immunocytochemically stained with Ki67 (dark brown; arrowheads) from LV^{SCR} treated (C) and $LV^{TLXShRNA}$ treated adolescent rats (D). Image taken at 10X magnification. Scale bar = 100 µm. Relative mRNA expression of DCX in the hippocampus of TLX knockdown and control adult rats. All values were adjusted to relative expression of the housekeeping gene *Actb* (E).

6.3.2 TLX knockdown during adolescence increased locomotor activity in adolescent but not adult rats

Adolescent rats that had been treated with TLX knockdown lentivirus ($LV^{TLXShRNA}$) travelled a significantly greater distance (cm) in the open field arena than control (LV^{SCR} treated) rats ($M_{LV}{}^{TLXShRNA}$ = 7061, $SD_{LV}{}^{TLXShRNA}$ = 1490; $M_{LV}{}^{SCR}$ = 5043, $SD_{LV}{}^{SCR}$ = 779; t (20) = 3.854, $p < 0.001$; Figure 6.4). TLX knockdown ($LV^{TLXShRNA}$ treated) adolescent rats also moved at a significantly greater velocity than the control (LV^{SCR} treated) rats ($M_{LV}{}^{TLXShRNA}$ = 12.01, $SD_{LV}{}^{TLXShRNA}$ = 2.54; $M_{LV}{}^{SCR}$ = 9.27, $SD_{LV}{}^{SCR}$ = 1.19; t (20) = 3.117, $p < 0.01$; Figure 6.4). During adulthood, there was a trend towards a decrease in the distance travelled in the TLX knockdown ($LV^{TLXShRNA}$) rats compared to control (LV^{SCR} treated) rats ($M_{LV}{}^{TLXShRNA}$ = 4041, $SD_{LV}{}^{TLXShRNA}$ = 1139; $M_{LV}{}^{SCR}$ = 4851, $SD_{LV}{}^{SCR}$ = 838; Figure 6.4) and there was no difference in velocity between the two groups (Figure 6.4).

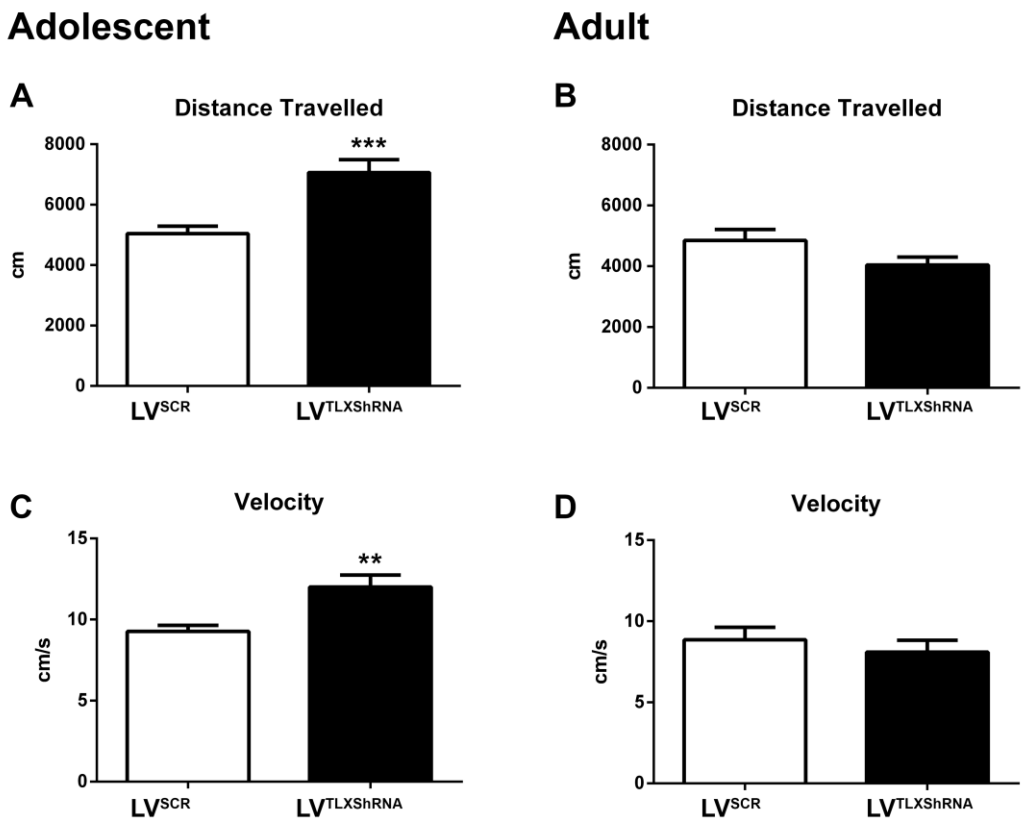


Figure 6.4: TLX knockdown during adolescence increased locomotor activity in adolescent but not adult rats

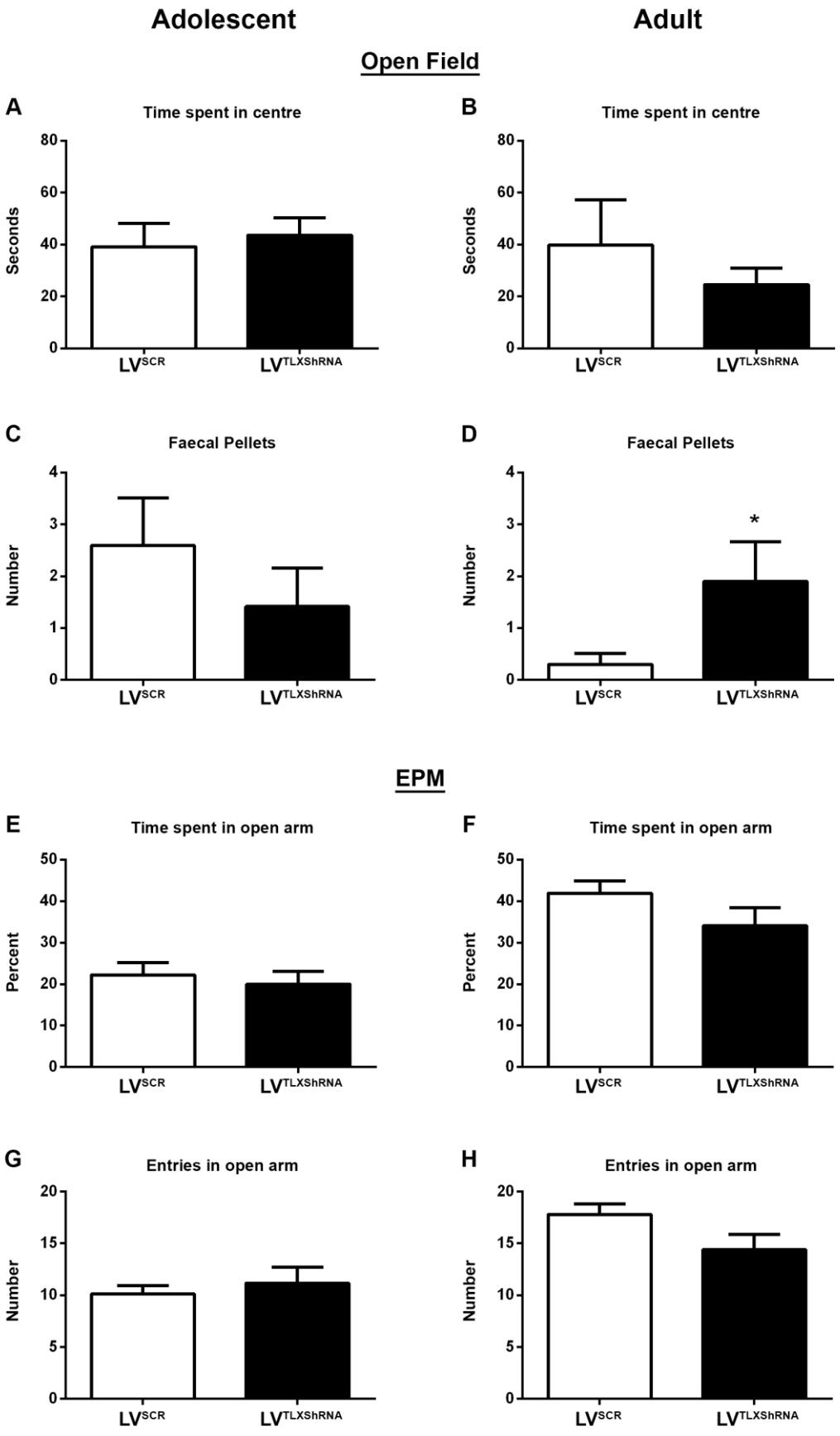
Distance travelled in the open field arena (cm) during adolescence (A) and adulthood (B) in LV^{SCR} treated (white bars) and $LV^{TLXShRNA}$ treated (black bars) rats. Velocity during exploration of the open field arena (cm/s) during adolescence (C) and adulthood (D) in LV^{SCR} treated (white bars) and $LV^{TLXShRNA}$ treated (black bars) rats. *** $p < 0.001$, ** $p < 0.01$; independent-sample t-test. All results are expressed as mean ± SEM. Sample size per age: adolescent (n = 10-12) and adult (n=10).

6.3.3 TLX knockdown during adolescence did not affect anxiety-related behaviours in adolescent rats but increased defecation in the open field in adult rats

Thigmotaxic behaviour remained the same across treatments and age; both adolescent and adult rats with or without TLX knockdown treatment spent similar time in the centre of the open field (Figure 6.5). Anxiety during the open field test was also measured by counting the number of faecal pellets in the arena after each animal had

been exposed to the test. TLX knockdown did not affect the number of faecal pellets in adolescent rats (Figure 6.5), but induced a significant increase in the number of pellets compared to the control (LV^{SCR} treated) group when assessed in adulthood (t (18) = 2.010, p = 0.05; Figure 6.5).

Anxiety-like behaviour in the EPM did not differ between adolescent TLX knockdown ($LV^{TLXShRNA}$) or control (LV^{SCR} treated) rats. Specifically, TLX knockdown ($LV^{TLXShRNA}$ treated) rats spent the same amount of time (percent of total) in the anxiolytic open arms of the maze as their control (LV^{SCR} treated) counterparts (Figure 6.5). Furthermore, the number of entries made to the open arms was the same across both treatment groups (Figure 6.5) as was the total number of entries made ($M_{LV}{}^{TLXShRNA}$= 27.75, $SD_{LV}{}^{TLXShRNA}$ = 8.65; $M_{LV}{}^{SCR}$ = 24.11, $SD_{LV}{}^{SCR}$ = 5.79). During adulthood, TLX knockdown did not affect the time rats spent in the open arm ($M_{LV}{}^{TLXShRNA}$ = 34.08, $SD_{LV}{}^{TLXShRNA}$ = 13.68; $M_{LV}{}^{SCR}$ = 41.94, $SD_{LV}{}^{SCR}$ = 9.37; Figure 6.5). Adult $LV^{TLXShRNA}$ treated rats showed trends towards reduced number of entries to the open arms ($M_{LV}{}^{TLXShRNA}$ = 14.4, $SD_{LV}{}^{TLXShRNA}$ = 4.71; $M_{LV}{}^{SCR}$ = 17.8, $SD_{LV}{}^{SCR}$ = 3.15; Figure 6.5) and reduced total number of entries ($M_{LV}{}^{TLXShRNA}$ = 29.7, $SD_{LV}{}^{TLXShRNA}$ = 7.71; $M_{LV}{}^{SCR}$ = 35.3, $SD_{LV}{}^{SCR}$ = 5.73).


Adolescent
Adult
Open Field
A
Time spent in centre
Seconds
80
60
40
20
0
LVSCR
LVTLXShRNA
B
Time spent in centre
Seconds
80
60
40
20
0
LVSCR
LVTLXShRNA
C
Faecal Pellets
Number
4
3
2
1
0
LVSCR
LVTLXShRNA
D
Faecal Pellets
Number
*
4
3
2
1
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LVSCR
LVTLXShRNA
EPM
E
Time spent in open arm
Percent
50
40
30
20
10
0
LVSCR
LVTLXShRNA
F
Time spent in open arm
Percent
50
40
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20
10
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LVSCR
LVTLXShRNA
G
Entries in open arm
Number
20
15
10
5
0
LVSCR
LVTLXShRNA
H
Entries in open arm
Number
20
15
10
5
0
LVSCR
LVTLXShRNA

Figure 6.5: TLX knockdown during adolescence did not affect anxiety-related behaviours in adolescent rats but increased defecation in the open field in adult rats
Thigmotaxis and number of faecal pellets in the open field as measures of anxiolytic behaviour during adolescence (time spent in centre: A, number of pellets: C) and adulthood (time spent in centre: B, number of pellets: D). Percent time spent in the open arms and number of entries to the open arms during the EPM task as measure of anxiety-like behaviour during adolescence (% time in open arm: E; number of entries to open arm: G) and during adulthood (% time in open arm: F; number of entries to open arm: H). White bars – LV^{SCR} treated rats, black bars – $LV^{TLXShRNA}$ treated rats. * $p < 0.05$; independent-sample t-test. All results are expressed as mean ± SEM. Sample size per age: adolescent (n = 10-12) and adult (n=10).

6.3.4 TLX knockdown increased immobility and reduced swimming frequency in adolescent rats, and increased swimming frequency in adult rats

There was no significant difference in performance between TLX knockdown ($LV^{TLXShRNA}$ treated) and control (LV^{SCR} treated) rats at adolescence or adulthood in the first triad of the forced swim test. Thus, during the first 5 minutes of the test, all animals displayed comparable mean scores on immobility frequency, swimming frequency, and climbing frequency (Figure 6.6). During the second triad, immobility frequency remained the same across treatments for both age cohorts (Figure 6.6). Similarly, climbing frequency did not differ across groups (Figure 6.6). There was no difference in swimming frequency between groups at adolescence (Figure 6.6), or adulthood (Figure 6.6). However, in the last 5 minutes of the test, adolescent TLX knockdown ($LV^{TLXShRNA}$ treated) rats exhibited a significantly lower score on swimming frequency behaviour (t (20) = 2.726, $p < 0.05$, Figure 6.6) which was coupled with a significant increase in immobility frequency (t (20) = 2.381, $p < 0.05$, Figure 6.6) compared to the control (LV^{SCR} treated) rats. Adult TLX knockdown ($LV^{TLXShRNA}$ treated) rats on the other hand, did not differ in their immobility score (Figure 6.6) but they did display a change in swimming frequency (t (18) = 2.242, p <

0.05) compared to controls (Figure 6.6). There was no change in the frequency of climbing behaviour across groups in this last phase of the test (Figure 6.6).

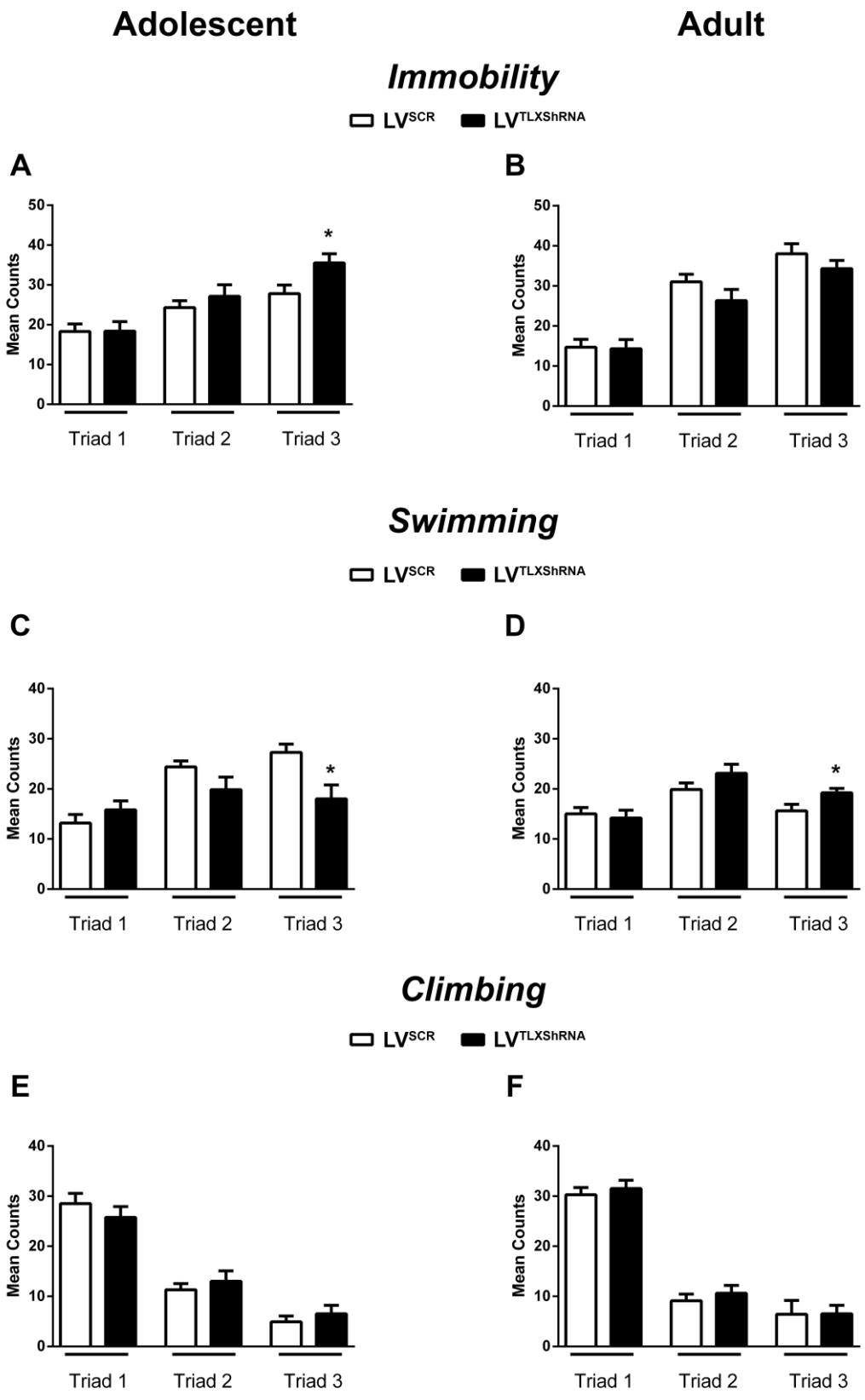

Figure 6.6: TLX knockdown increased immobility and reduced swimming frequency in adolescent rats, and increased swimming frequency in adult rats in the forced swim test

Active behaviours in the forced swim test for Triad 1 (0-5 min), Triad 2 (5-10 min) and Triad 3 (19-15 min). Immobility behaviour frequency for adolescent (A) and adult (B) rats, swimming behaviour frequency for adolescent (C) and adult (D) rats, and climbing behaviour frequency for adolescent (E) and adult (F) rats treated with LV^{SCR} (white bars) or $LV^{TLXShRNA}$ (black bars). * $p < 0.05$; independent-sample t-test. All results are expressed as mean ± SEM. Sample size per age: adolescent (n = 10-12) and adult (n=10).

6.3.5 TLX knockdown during adolescence did not affect performance in the pattern separation task or the contextual and cued fear conditioning paradigm in adolescent or adult rats

$LV^{TLXShRNA}$ treatment had no effect on performance in the pattern separation task by adolescent rats in either the large separation or the neurogenesis-associated small separation (Figure 6.7). Pattern separation refers to the ability to distinguish and store similar inputs as distinct representations in memory (Hvoslef-Eide and Oomen, 2016). One-sample t-test revealed that neither of the groups performed significantly different from chance level in both versions of the test. No difference in performance on the large separation task was observed between adult rats treated with TLX knockdown ($LV^{TLXShRNA}$) or control (LV^{SCR} ; Figure 6.7). In addition, neither group could discriminate between the novel and familiar locations as determined by one-sample t-test comparing the average of each group to chance performance. There was no change in the performance of adult rats treated with TLX knockdown ($LV^{TLXShRNA}$ treated) compared to control (LV^{SCR} treated) rats in the small separation task (Figure 6.7). The performance of both groups was at chance level as determined by an one-sample t test.

No changes in the freezing behaviour of TLX knockdown ($LV^{TLXShRNA}$ treated) rats were observed during the context test of the fear conditioning paradigm. Both adolescent and adult TLX knockdown ($LV^{TLXShRNA}$ treated) rats were motionless for similar proportion of time as the control (LV^{SCR} treated) rats (Figure 6.7). The Mauchly's test indicated that the assumption of sphericity had been violated for the cued fear conditioning data for the adolescent cohort (χ^2 (2) = 15.024, $p < 0.01$). Therefore, the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity as the epsilon was less than 0.75 ($\varepsilon = 0.707$). The results showed that rats in both treatment groups maintained a high percent freezing time throughout the presentation of all three tones (Figure 6.7). Furthermore, rats treated with with TLX knockdown ($LV^{TLXShRNA}$) exhibited similar cued fear recall response as the control (LV^{SCR} treated) rats (Figure 6.7). There was no within-subject effect of the repeated tone presentations on freezing behaviour in the adult cohort (Figure 6.7). Lastly, both groups retained the same level of cued fear recall (Figure 6.7).

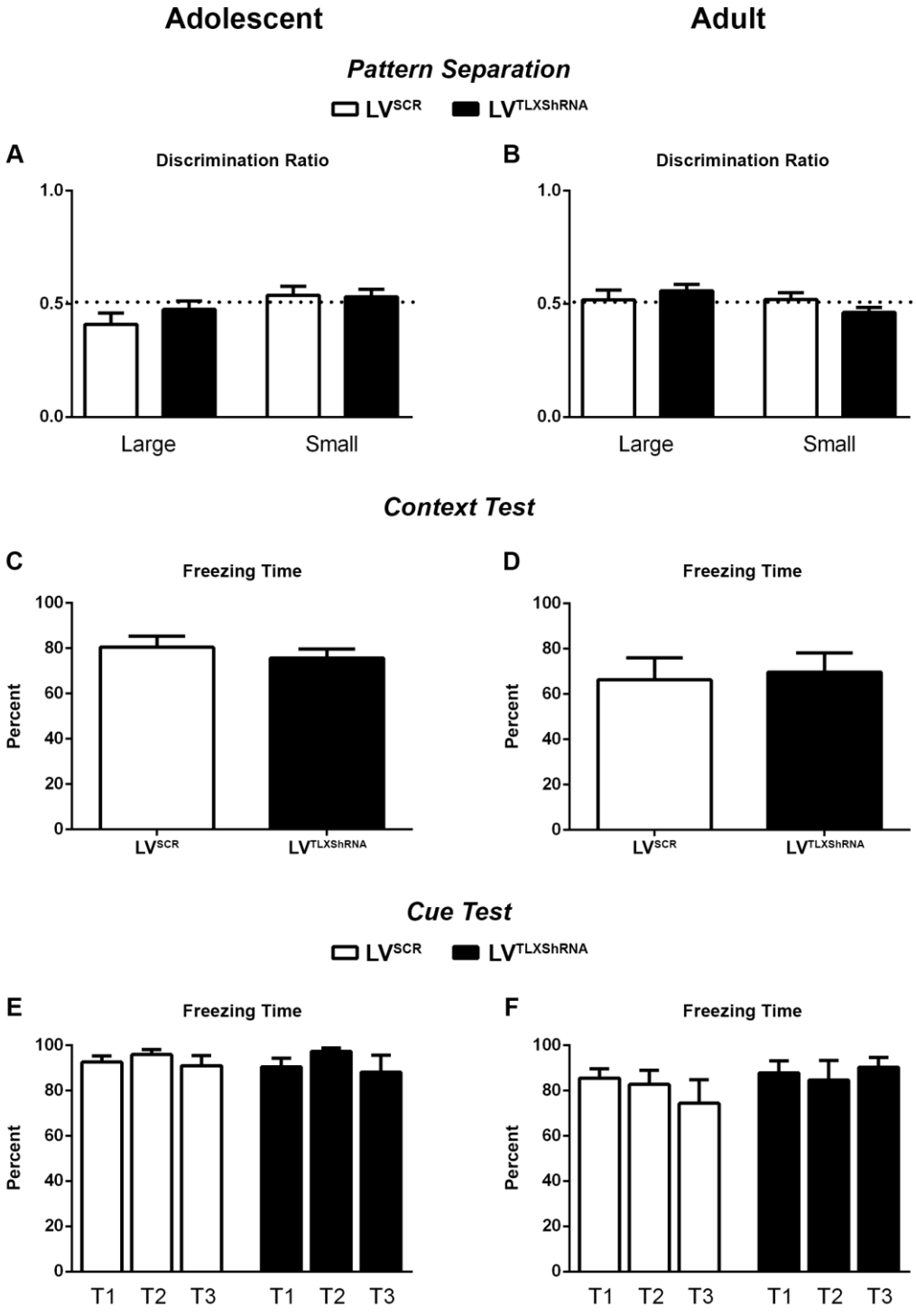

Figure 6.7: TLX knockdown did not affect performance in pattern separation and contextual and cued fear conditioning during adulthood and adolescence.
Performance on the large and small pattern separation task during adolescence (A) and adulthood (B). Contextual freeze behaviour in the fear conditioning paradigm during adolescence (C) and adulthood (D). Cued fear conditioning during adolescence (E) and adulthood (F). All results are expressed as mean ± SEM and were analyzed with an independent-sample t-test, except in (E) and (F) when repeated measures ANOVA was employed followed by Bonferoni post hoc analysis. Data in (A) and (B) were

also compared against chance level (dashed line) by one-sample t-test. Sample size per age: adolescent (n = 10-12) and adult (n=10). White bars – LV^{SCR} treated rats, black bars – $LV^{TLXShRNA}$ treated rats.

6.3.6 TLX knockdown during adulthood did not impair behaviour in the Y-maze

Spontaneous alternation behaviour, a measure of spatial working memory, was tested in adult rats treated with TLX knockdown ($LV^{TLXShRNA}$.). TLX knockdown showed no significant effect on the alternation rates when compared to the control (LV^{SCR} treated) rats (Figure 6.8). Furthermore, both groups exhibited a comparable number of arm entries (Figure 6.8) indicating similar motivation states and total activity in all animals. Since the alternation rates for both TLX knockdown ($LV^{TLXShRNA}$ treated) and control (LV^{SCR} treated) rats did not reach the criteria of 75% (Deacon and Rawlins, 2006), the modified discrete or rewarded alternation test of working memory was performed in the Y-maze. TLX knockdown had no influence on the percent of correct choices made in the discrete alternation immediately after (Figure 6.8) or one minute after (Figure 6.8) an animal had undergone the sample/forced-run phase.

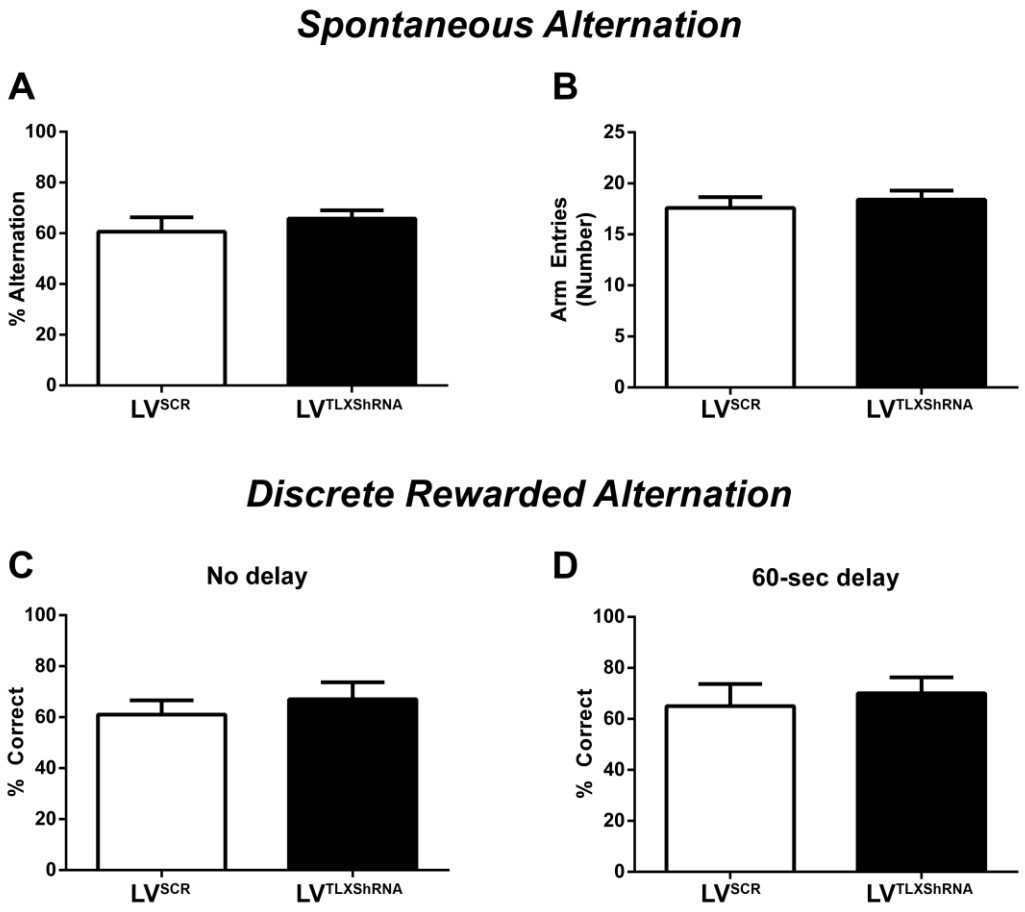

Figure 6.8: TLX knockdown during adulthood did not affect behaviour in the Y-maze.
Percent spontaneous alternation in the Y-maze (A) during adulthood and number of arm entries (B) for LV^{SCR} treated (white bars) and $LV^{TLXShRNA}$ treated (black bars) adult rats. Percent correct choices made during the discrete rewarded alternation task in the Y-maze without (C) or with (D) 60-second delay in LV^{SCR} treated (white bars) and $LV^{TLXShRNA}$ treated (black bars) adult rats. All results are expressed as mean ± SEM and were analyzed with an independent-sample t-test. n=10 in each group.

6.4 Discussion

The results from these experiments show that lentiviral transduction successfully diminished TLX protein expression in the rat DG, which was associated with a decrease in the number of proliferating cells in the dGCL. Secondly, we show that TLX knockdown during adolescence induced hyperactivity in adolescent but not adult rats. With respect to anxiety-related behaviours measured by EPM and thigmotaxis in the open field, we found no differences between groups across the two age cohorts.

However, there was a significant increase in defecation in the open field arena by $LV^{TLXShRNA}$ treated adult rats. The active behaviours in the forced swim test did not differ until the final triad or the last 5 minutes of the 15-minute test. During this time frame, we observed an increased frequency in immobility behaviour and decreased frequency in swimming behaviour by $LV^{TLXShRNA}$ treated rats compared to the LV^{SCR} treated controls during late adolescence. On the contrary, $LV^{TLXShRNA}$ treated animals exhibited increase in swimming frequency in adulthood. TLX knockdown did not hinder performance on the pattern separation task or the contextual and cued fear conditioning paradigm, neither during adolescence, nor adulthood. Additionally, performance on the spontaneous and discrete alternation in the Y-maze during adulthood was also not affected by silencing of the TLX gene. Thus, these data confirm that targeted disruption of the TLX gene within the dDG during early adolescence can have differential effects on behaviour during late adolescence and adulthood, without affecting learning and memory at either age. The current data thus highlight the temporal nature and function of the nuclear receptor TLX during development.

Lentiviral vectors are an appealing vehicle for shRNA delivery whereby stable knockdown of a gene has been achieved with high efficiency in many different mammalian cell types (Brown et al., 2010; Frank et al., 2017). Here we deliver a lentiviral shRNA vector designed for mouse TLX gene into the rat hippocampus. The gene homology for TLX between species is very high with 100% identity in the DNA-binding domain and 98% identity in the ligand-binding domain between mouse and rat (Yu et al., 1994). The high homology across species has also been demonstrated in

a study where the aggressive phenotype of mice with spontaneous deletion of the gene was rescued by expression of its human homologue (Abrahams et al., 2005). The efficiency of the knockdown was confirmed by a decrease in fluorescence intensity of immunohistochemically labelled TLX protein in the adolescent cohort. TLX is a major regulator of NSC proliferation (Sun et al., 2007). We confirmed this role of TLX in the hippocampus by examining the density of Ki67+ cells in $LV^{TLXShRNA}$ treated rats and showed that there was a significant reduction in the number of proliferating cells in the dDG but not the vDG. It is important to note that neurogenesis is a multifactorial process of cell proliferation, differentiation and neuronal integration. Thus it may be possible that knocking down one of these factors alone (as in this case TLX knockdown targets proliferating NPCs) is not sufficient to enhance hippocampal-associated behaviour or the other stages of neurogenesis such as differentiation and neuronal integration. Additionally, inhibiting one specific component of neurogenesis may not only dysregulate the entire process, but also may not translate into decrease in differentiation and/or integration of the newborn cells. For instance, it has been shown that overexpressing the transcription factor Sonic Hedgehog (Shh) in NPCs, resulted in increased cell proliferation but no change in number of differentiating neurons (Lai et al., 2003). Similarly, we only observed a trend towards, rather than a significant decrease in the expression of hippocampal DCX in our adult cohort. It has been demonstrated that TLX prevents NPC differentiation by sustaining them in a proliferative state (Li et al., 2008; Shi et al., 2004; Niu et al., 2011). Our behavioural and immunohistochemical data suggest that despite the decreased cell proliferation within the dorsal DG, brought about by TLX knockdown, did not result in a significant decrease in neurogenesis overall. Rather, it appears that inhibiting the proliferative

state of NPCs within the dorsal DG was achieved without significantly altering the differentiation status or net outcome number of newborn neurons. This is in line with the work on Shh by Lai et al. (2003), which could also explain why we did not observe any differences between controls and rats treated with TLX shRNA in the pattern separation task, contextual fear conditioning task or the spontaneous alternation in the Y-maze, as there may be no significant decrease in the number of functional newborn granule cells. It further reinforces the concept that for any impairment in hippocampal cognitive function to be induced by altering NPC state, there must be a robust inhibition of all stages of neurogenesis.

Silencing TLX expression in the juvenile rat dDG was associated with a hyperactive phenotype during late adolescence but not adulthood. We and others have previously shown that mice with spontaneous deletion of the TLX gene display hyperactivity during adolescence and adulthood (O'Leary et al., 2016a; Wong et al., 2010; Young et al., 2002). When TLX was silenced in the hippocampus of two-month-old mice by inducible recombination and locomotor behaviour was examined later in adulthood, no hyperactivity or motor impairments were observed (Zhang et al., 2008), which corroborates our findings from the adult rat cohort of the present study. Thus, in the absence of TLX from the embryonic period hyperactivity occurs throughout life, when TLX is silenced during the juvenile period hyperactivity only manifests for short period after the knockdown of the gene, and when TLX is silenced in adulthood the hyperactive phenotype is not present. Interestingly, in another mouse model where TLX was knocked out prior to E12 through homologous recombination, hyperactivity

was not observed despite the presence of impairments in forebrain development and deficiencies in emotional behaviour (Roy et al., 2002). Remarkably, the impairments observed in the homologous recombination model matched those observed in the spontaneous deletion model except that an intact striatum was evident in the former but not the latter mouse model (Drill, 2009; O'Leary et al., 2016a; O'Leary et al., 2016b; Roy et al., 2002; Young et al., 2002). The striatum plays a crucial role in the differential processing of reward-seeking and risk-taking behaviour during adolescence and adulthood (Galvan, 2010; Sturman and Moghaddam, 2012), which may be modulated by differential pruning of dopamine receptors (Teicher et al., 1995). Due to the connectivity between the hippocampus and striatum (Ghiglieri et al., 2011; Machado et al., 2014), it is possible that the targeted silencing of TLX in the dDG of the juvenile rats in the present study caused changes to striatal signalling and function, which resulted in hyperactivity. Given that development of the striatum undergoes crucial anatomical changes between adolescence and adulthood it is also possible that the adolescent striatum is more susceptible to TLX knockdown in the dDG than the adult. It may also be that silencing of TLX in the juvenile dDG induced a transient effect, and that compensatory mechanisms are taking place during adolescence so that the hyperactive phenotype is overcome during adulthood.

We did not observe an effect of TLX knockdown on anxiety-related behaviours in either late adolescence, or during adulthood. While we previously observed a thigmotaxis phenotype in mice with a spontaneous deletion of TLX both during adolescence and adulthood (O'Leary et al., 2016a), there was no evidence of this

behaviour in the current study using rats with targeted silencing of the gene within the dDG. Furthermore, there was no change in performance by $LV^{TLXShRNA}$ treated rats in the EPM during adolescence or adulthood, while mice with TLX knockout through homologous recombination have been reported to exhibit an increase in anxiogenic behaviour in the EPM (Roy et al., 2002). This discrepancy in findings may be explained by the fact that the vDG is primarily involved in processing and regulation of anxiety-related behaviours (Levone et al., 2015; O'Leary and Cryan, 2014). In the current study TLX was knocked down only in the dDG of the rats, while in both studies showing anxiolytic behaviour in mouse models, TLX was constitutively knocked out (O'Leary et al., 2016a; Roy et al., 2002).

We observed an increase in defecation behaviour in the $LV^{TLXShRNA}$ treated group compared to control during adulthood, but not adolescence. However, on comparing the defecation behaviour from both adult and adolescent cohorts, the LV^{SCR} treated adolescents exhibit an increase in defecation compared to their adult counterparts. This is in line with the fact that the propulsive capacity of the colon is reduced over time and that gastric digestion slows down with age (Madsen and Graff, 2004). Though measuring defecation and urination has previously been used as a record of anxiety-like behaviour in rats (Crumeyrolle-Arias et al., 2014; Monnikes et al., 1993), it has been disputed as less reliable than thigmotaxis with reports on differences in baseline defecation level dependent on weight, sex and strain (Russell, 1973; Walsh and Cummins, 1976). Nonetheless, the behavioural outputs from the anxiety-related tests showed no change in response to TLX knockdown, while the defecation response

revealed increased anxiety-like behaviour in the $LV^{TLXShRNA}$ treated group in the stressful environment of the Open Field. This finding is particularly curious in the light of the studies showing reduction in anxiety-like behaviour in mice with homologous recombination or spontaneous deletion of the TLX gene for both the Open Field test and EPM (Roy et al., 2002; Young et al., 2002). To the best of our knowledge, the autonomic response to stress in these mouse models has not been measured to date. It would be thus interesting to examine whether the autonomic and behavioural outputs align when TLX expression is absent from utero.

Examination of behaviour during the FST showed that during the first two triads of the 15-min test there were no differences in the frequency of active and passive behaviours between LV^{SCR} and $LV^{TLXShRNA}$ treated rats from either age cohort. Remarkably, during the third triad of the FST, a marked increase in immobility behaviour, coupled with a decrease in swimming behaviour frequency was observed in the $LV^{TLXShRNA}$ treated adolescents compared to the LV^{SCR} treated controls during late adolescence. This is particularly interesting, given that during adolescence the $LV^{TLXShRNA}$ treated rats were hyperactive. On the contrary, adult $LV^{TLXShRNA}$ treated rats exhibited an increase in swimming behaviour compared to their LV^{SCR} treated counterparts. Exposure to the FST has been shown to activate a multitude of systems and circuits including the sympathetic nervous system, the HPA as well as the dopamine- and serotonin- neurotransmitter systems (de Kloet and Molendijk, 2016). Furthermore, the depressive-like phenotype induced by FST has been shown to be reversible after administration of various serotonergic agonists, reuptake inhibitors

and transporters (Cryan et al., 2005; Detke et al., 1995; Ulloa et al., 2014). Additionally, serotonin receptors have been implicated in the regulation of the aggressive phenotype of the mice with spontaneous deletion of TLX (Juarez et al., 2013). Future studies should investigate the serotonin-TLX interaction in terms of mechanism and dynamics throughout development.

There was no effect of TLX knockdown in the dDG of juvenile rats on cognitive performance during either late adolescence or adulthood. We did not observe any differences in the performance of control or TLX-knockdown rats in the large separation task, or in the neurogenesis-associated smaller and more difficult separation task (Bekinschtein et al., 2014). What is more, in both tasks and both age cohorts, exploration behaviour of the objects was at chance level. It is possible that presence of the virus itself in the dDG of both LV^{SCR} and $LV^{TLXShRNA}$ cohorts of animals rendered the acquisition of this hippocampal-dependent task too difficult. We used contextual and cued fear conditioning to assess the rodents' ability for associative learning and episodic memory recall (Curzon et al., 2009). With this test acquisition could be controlled for more robustly and indeed we saw that all animals learned to associate the unconditioned stimulus (US; shock) with the conditioned stimulus (CS; tone; data not shown) during the acquisition phase of the test. However, TLX knockdown did not affect the ability of the animals to recall, during both adolescence and adulthood. This contrasts to the results obtained from mice where TLX was knocked out through homologous recombination (Roy et al., 2002) and in a study where we previously showed that mice with a spontaneous deletion of TLX displayed

impaired performance in contextual fear conditioning during adolescence but not adulthood (O'Leary et al., 2016a). However, in a mouse model with conditional deletion of TLX during adulthood, contextual fear conditioning in the absence of TLX was normal (Zhang et al., 2008), corroborating our findings in rats. These data thus suggest that disrupting TLX during early neurodevelopment affects hippocampus-dependent learning and memory as measured by contextual fear conditioning during adolescence but not later in adulthood, and disruption of TLX during juvenile development does not impair learning and memory as measured by this paradigm. The amygdala-dependent cued fear conditioning response also remained the same between LV^{SCR} and $LV^{TLXShRNA}$ treated rats assessed in adolescence and adulthood. A mouse model employing targeted deletion of TLX in adulthood complements the present findings with respect to showing no difference in cued fear recall between controls and mice lacking TLX from 8 weeks of age (Zhang et al., 2008). In mouse models of TLX deletion during early development however, adult and adolescent TLX knockout mice performed poorer than their control littermates in cued fear conditioning (O'Leary et al., 2016a; Roy et al., 2002). It is possible that the lack of effect of TLX knockdown on cued fear recall was due to the small number of tones presented. Here only 3 tones were presented, while others have been performing this task using 15 or even up to 50 tones to measure cued fear recall (Curzon et al., 2009). Taken together, the results of contextual and cued fear conditioning from the various models of TLX deletion imply that in the absence of TLX during early life, key limbic system structures such as the hippocampus and amygdala may develop abnormally and result in impairment in associated behaviours, while the same does not occur as a result of deletion of TLX during adolescence or adulthood. We found no impairment in

spontaneous alternations in the Y maze as a result of $LV^{TLXShRNA}$. In mice with a spontaneous deletion of TLX from utero, a decrease in the percent of alternations during adolescence, but not during adulthood, was observed (O'Leary et al., 2016a). Likewise, we did not observe any impairment in the performance of adult $LV^{TLXShRNA}$ treated rats on the rewarded or discrete alternation in the Y maze. Despite the crucial involvement of the septal-hippocampal system in this task, other brain areas have also been shown to play a significant role (Deacon and Rawlins, 2006; Lalonde, 2002), which may have compensated for a lack of TLX in the dDG.

6.5 Conclusions

Our results illustrate changes in behaviours as a result of TLX knockdown in the dorsal hippocampus. TLX is expressed on stem cells (Benod et al., 2016) and primarily controls the proliferative capacity of NPCs (Islam and Zhang, 2015). Although silencing TLX led to reduced cell proliferation in the dDG, it may have not influenced the function of new-born neurons and associated cognitive processes as assessed by the tasks in the current study and in the time frame of the study. The manifestation of behaviours such as hyperactivity and a depressive-like phenotype in adolescence but not adulthood in response to TLX knockdown during the juvenile period suggest that compensatory mechanisms may be taking place during adolescence for these behavioural phenotypes. However, a limitation of the present study is the lack of experimental group where TLX expression has been silenced during adulthood alone. In order to confirm the temporal effect of TLX during development in future studies, it would be important to perform an experiment employing adult lentiviral delivery of TLX shRNA to the dDG of the rat. Future studies investigating the temporal

consequences of TLX knockdown on such behaviours are now warranted, including studies examining a sex-dependent effect of TLX during development and adulthood. Conversely, the differential effects of TLX knockdown on swimming behaviour in the FST and defecation behaviour between adolescent and adult rats suggest a potential age-related role for TLX in stress-induced behaviour and in the autonomic-regulated response to a stressful environment. Further investigation may aid deeper understanding of the role of TLX and parallel pathways to it in neurobiological disorders and their potential treatment.

Chapter 7

General Discussion

7.1 Overview and aims

A number of neurological disorders (for instance PD, stroke, multiple sclerosis, spinal cord injury) present a common etiological pattern of development that begin with the loss of neuronal and glial cells (Lindvall and Kokaia, 2006). There is a growing demand for developing novel therapies and methods to alleviate symptoms for Alzheimer's disease and dementia, and a lot of attention has focused on targeting the endogenous NSCs in the brain to use for repair and for repopulating the areas affected by neuronal loss (Duncan and Valenzuela, 2017; Lindvall et al., 2004; Tang, 2012). For example, in the last two decades, great advancements have been made for the treatment of spinal cord injuries with stem cell based therapies (Nandoe Tewarie et al., 2009; Schroeder et al., 2016; Vismara et al., 2017). The superfamily of nuclear receptors is involved in a diverse range of biological processes and due to their structure (DNA-binding and ligand regulated) as well as function and relevance to many diseases (for instance cancer prognosis and progression of various types of cancer; (Aesoy et al., 2015) and see the special issue from Acta Pharmacologica Sinica, January 2015 on Nuclear receptors: biology, structure and drug discovery), they have become important therapeutic targets (Shi, 2007). One such receptor and the focus of the present work is the orphan nuclear receptor TLX. It has prompted a lot of excitement in the last decade, given that it was termed a master regulator of NSCs (Islam and Zhang, 2015). It maintains the stem cell pool in the adult neurogenic niches, and it has been thought that TLX presents a valuable therapeutic target for neurodegenerative diseases (Sobhan and Funa, 2017; Sun et al., 2017). The main goal of this work was to add to the knowledge and understanding of the functions and

mechanisms of action of TLX and therefore to position and aid in its development for neurodegenerative-related therapies.

The primary aims of the present work were three-fold. Firstly, we investigated whether TLX plays a role in the interaction between hippocampal NS/PCs and the resident immune cells, microglia, and thus we examined whether changes in microglial phenotype and activation state occurred in mice lacking TLX expression, or whether changes in NSC regulators occurred in mice where microglia were impaired. Secondly, we assessed whether TLX had an impact on baseline hippocampal neurogenesis and exercise-induced neurogenesis during the critical period of adolescence. Given that the period of adolescence is vulnerable to the effects of stress and social isolation, we examined whether social isolation influences the neurogenic outcome during this time. Lastly, in order to further characterize the role of TLX during adolescence we examined whether a lack of TLX at this time played a role in cognitive and anxiety-related behaviours. We used two different models of TLX deletion in order to validate our results. We also compared the behavioural outcomes from adolescence with those from adulthood to determine whether there was a critical period for TLX function.

7.2 TLX and microglial-neuronal crosstalk

We demonstrated that in the absence of TLX, an activated microglial phenotype correlates with an increased level of endogenous IL-1β and impaired hippocampal neurogenesis. Thus, we are the first to implicate TLX as an important potential

mediator of NS/PC-microglial communication. However, the temporal relation between these associations remains unclear. For instance, one possibility is that lack of TLX expression on NSCs has prompted astrocytes to release IL-1β and that the increased levels of the cytokine in turn have provoked microglia to assume an activated phenotype. Alternatively, another possible explanation is that in the absence of TLX, NS/PC secrete pathological signals to microglia which brings about their activation and increased IL-1β production independently of astrocytes. Lastly, it is possible that the observed correlations occur autonomously of each other and that TLX is not part of the pathway linking NS/PCs signals to the immune system. To resolve these issues in future studies, it would be important to establish what is the source of elevated IL-1β in the hippocampi of mice lacking TLX expression. Insight into this could be obtained by isolating microglia from the hippocampi of $Nr2e1^{-/-}$ mice and examining how these microglia behave in response to pro- and anti-inflammatory stimulation with different cytokines, or whether microglia are responsible for the observed IL-1β-induced increase. Additionally, microglia primed towards a pro-neurogenic phenotype through exercise for instance, have been shown to have positive effects on NPC cultures. Thus it would be very interesting to check what effect microglia isolated from TLX deficient mice would have on hippocampal NPC cultures (de Miranda et al., 2017). This would provide insight into whether microglia in the absence of TLX have developed an anti-neurogenic phenotype or whether they assume normal physiological function when presented to NPCs that express TLX.

In order to further test our hypothesis about the involvement of TLX in the signalling between NS/PCs and microglia, we examined whether TLX expression was affected in a model where this interaction was impaired, namely the CX3CR1 knockout mouse model. The $CX3CR1^{KO}$ mice lack the fractalkine receptor that is important for detecting signal inputs from neurons in the surrounding environment and from hippocampal NPCs in particular (Lauro et al., 2015; Limatola and Ransohoff, 2014; Maggi et al., 2011; Pagani et al., 2015). We observed a reduced genetic transcription of TLX in the hippocampi of $CX3CR1^{KO}$ mice, which further supported a possible role of the nuclear receptor in the signalling pathway between NS/PCs and microglia. More importantly, our data showed that upstream regulators of TLX were not affected. Rather, the lack of CX3CR1 was associated with downregulation of TLX and upregulation of some of its target genes. Our results were correlational only but provide a plausible explanation for the observed decrease in neurogenesis reported for $CX3CR1^{KO}$ mice. We speculate that since the reduction of neurogenesis associated with CX3CR1 deficiency (Bachstetter et al., 2011; Rogers et al., 2011; Xiao et al., 2015) occurs independent of Sox2 (based on our data, Chapter 3), it is possible that regulation occurs through TLX-dependent signalling pathways. To further examine the bidirectionality of this association, it would be important to test whether $Nr2e1^{-/-}$ mice exhibit impaired CX3CL1 secretion by hippocampal granule cells. A major limitation of our study in Chapter 3 is that gene expression only was examined. It would be important to perform protein analysis of the hippocampi of $CX3CR1^{KO}$ mice and to isolate hippocampal NPCs to see how they behave *in vitro*, and perhaps also to compare with primary cultures from $Nr2e1^{-/-}$ mice. Additionally, we have not examined here whether the complement system plays a role in either knockout model.

The complement system, part of the immune system, consists of a group of proteins expressed by microglia, astrocytes and neurons and has been shown to be a major regulator of CNS homeostasis as well as of synaptic pruning during development (Presumey et al., 2017; Stephan et al., 2012). Thus, to better characterize the Nr2e1$^{-/-}$ and the CX3CR1^{KO} mice the function of the complement system should be investigated. Figure 7.1 summarizes findings from our lab and others regarding the two knockout models and the questions that remain to be answered. One such question is whether neurons generated from hippocampal NS/PCs in the absence of TLX are the culprits of microglial activation observed in Nr2e1$^{-/-}$ mice. Albeit dramatically reduced in number and presenting an impaired morphology, we still found newborn neurons in the DG of mice lacking TLX (see chapter 2). Thus it is possible that it is not the NS/PCs themselves but rather the newborn neurons that release factors triggering the pro-inflammatory environment and activation phenotype of microglia.

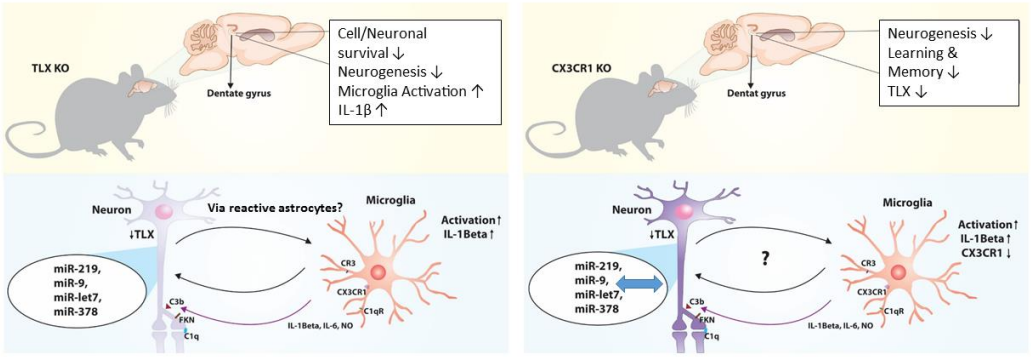

Figure 7.1: Summary of Nr2e1-/- and CX3CR1KO mice in the context of neurogenesis and microglial phenotype.

Since it has been shown that both Nr2e1$^{-/-}$ and CX3CR1^{KO} mice present hippocampal, but not olfactory, impaired memory performance (de Miranda et al., 2017; Reshef et al., 2014; Rogers et al., 2011), it would be of interest to examine TLX expression in the SVZ of the CX3CR1^{KO} mice. Additionally, we would obtain specific data if we

dissect the DG in future experiments, so that we eliminate the confound of examining the molecular environment in the hippocampus as a whole as opposed to the neurogenic region per se. To the best of our knowledge we are the first to tap into the possible mechanisms underlying the impaired neurogenesis observed in CX3CR1^{KO} mice, but our experiment was not exhaustive in terms of the molecular players analyzed. Other pathways involved in the regulation of neurogenesis (See Table 1.1) may also contribute to the reduced neurogenesis and survival reported in CX3CR1^{KO} mice (de Miranda et al., 2017; Reshef et al., 2014).

7.3 Exercise, isolation stress and TLX in the context of adolescence

In the light of decreased neurogenesis displayed by Nr2e1$^{-/-}$ mice and the proneurogenic effects of exercise widely reported in the last decades (van Praag, 2008; van Praag et al., 1999a; van Praag et al., 1999b; Vukovic et al., 2012), we examined whether voluntary running could ameliorate the neurogenic deficit in adolescent Nr2e1$^{-/-}$ mice. We focused on the adolescent period for several reasons. Firstly, adolescence is a critical period of brain development characterized by cortical growth and the establishment of many new synaptic connections(Crews et al., 2007). Thus, we hypothesized that it may be optimal to challenge the deficits caused by lack of TLX during adolescence due to the increased neuronal plasticity reported in normal developing adolescent rodents (He and Crews, 2007; Hueston et al., 2017). Secondly, adolescence is a critical period of vulnerability to developing stress-related disorders as well as other psychiatric conditions such as bipolar disorder. Moreover, adolescence is a highly susceptible time to positive and negative environmental influences, a period

of change and transition (Hueston et al., 2017; Jaworska and MacQueen, 2015; Medicine, 1999). Thirdly, we observed (Chapter 5) that adolescent mice lacking TLX presented with a greater degree of cognitive impairments than adults. Thus, we hypothesized that exercise during adolescence may improve the rate of neurogenesis in TLX deficient mice. However, the data actually demonstrated that TLX is necessary for the pro-neurogenic effects of exercise to occur. Interestingly, CX3CR1 has also been demonstrated as a necessary mediator of the pro-neurogenic effects of exercise (de Miranda et al., 2017). This suggests that the homeostasis between NS/PC-microglial signalling needs to be in place for the stimulatory effects of exercise to become evident. It has been proposed that the beneficial effects of running are exerted through BDNF signalling (Gomez-Pinilla et al., 2008; Liu and Nusslock, 2018; Marosi and Mattson, 2014; Sleiman et al., 2016). It is possible that in both mouse knockout models investigated in this thesis ($Nr2e1^{-/-}$ and $CS3CR1^{KO}$) the production and secretion of BDNF is blocked or impaired. Thus, it would be interesting to test whether administering exogenous BDNF in combination with an exercise regime in the absence of TLX or CX3CR1 would stimulate the proliferation and differentiation of NPCs. It has been shown that the environment can have influence on behaviour and molecular mechanisms in the brain (DePasquale et al., 2016), but whether environmental stimulation can overcome genetic impairments remains to be tested.

Another interesting finding that emanated from this study (Chapter 4) came from our wildtype control groups. Due to the aggressive nature of the male $Nr2e1^{-/-}$ mice they needed to be single-housed throughout the study. We compared the data obtained from

them to data from single-housed wildtypes. Since social isolation has been shown to be a stressor (Cruces et al., 2014; Ieraci et al., 2016; Stranahan et al., 2006) and given the fact that adolescence can be a sensitive period when sociability is heightened (Liu et al., 2015; Skelly et al., 2015), we compared the data from the single-housed wildtype mice to pair-housed mice with or without access to a running wheel. We discovered that social isolation attenuated the pro-neurogenic effects of exercise preferentially in the vDG, a region involved in anxiety processing and the stress response (O'Leary and Cryan, 2014). Social isolation as a rodent stress model during adolescence has strong validity and is considered highly translatable to human studies (Burrows et al., 2011; Koolhaas et al., 2017). Hence, it would be important to confirm our findings (Chapter 4) in a study of human adolescents in the context of exercise and social isolation. For instance a recent study conducted with 20-year-old humans, employing high resolution MRI imaging and a pattern separation task, found that a rapid bout (10-min) of physical activity had enhancing effects on performance on the neurogenesis-associated pattern separation task and increased functional connectivity between hippocampal (DG/CA3) and cortical regions (Suwabe et al., 2018). Moreover, the authors found that the magnitude by which connectivity was strengthened was predicitive of performance on the pattern separation task on an individual level (Suwabe et al., 2018). In the context of our findings from Chapter 4, several questions (albeit largely speculative) regarding translatability to human studies arise: is there an implication regarding individual and team sports – i.e. examine whether engaging in a sport activity during adolescence has an effect on brain connectivity and performance on a cognitive tasks such as the pattern separation employed by (Suwabe et al., 2018); if so is there a difference between engaging in an individual or team sport activity; is there

a difference in the brain response between teenagers that are popular/have a large group of friends and teenagers that have been outcast by their peers?

7.4 Cognitive performance during adolescence and adulthood in the presence/absence of TLX

In line with our continuous examination of whether TLX would be a valid therapeutic target for neurological conditions where NSC modulation may be beneficial, we further characterized the behavioural phenotype of the TLX knockout mice. To this end we performed a battery of tests across all three genotypes ($Nr2e1^{-/-}$, $Nr2e1^{+/-}$ and wildtypes), with both male and female mice during adolescence and subsequently during adulthood. We found that TLX deficiency was associated with impairments in motor, cognitive and anxiety-related behaviour in both male and female mice. Interestingly, the majority of effects occurred during adolescence and by adulthood had disappeared, again highlighting the sensitivity of the adolescent period to disruption in TLX signalling and NS/PC maintenance. It is important to note that not all behaviours that presented with impairment were hippocampal-dependent. This is potentially due to the fact that in the $Nr2e1^{-/-}$ model of spontaneous deletion of TLX, many developmental abnormalities in specific brain areas take place and parts of the brain remain underdeveloped or smaller in size (Young et al., 2002). It was surprising to observe that the impairments in certain behaviours did not persist into adulthood, such as performance on the contextual fear conditioning paradigm, while with other tests, performance deteriorated with age, such as motor performance on the rotarod. With respect to cognitive performance on the contextual fear conditioning tasks, no impairments were observed with the female cohort of animals. It is important to note

that male Nr2e1$^{-/-}$ mice were single housed after weaning due to their aggressive nature, while the Nr2e1$^{-/-}$ females were group housed. This confounding factor may have contributed to the impoverished performance of the adolescent males. To overcome this caveat, future studies ought to consider single-housing females also. The same cohort of animals was used for behavioural testing during adolescence and adulthood in line with the replacement, reduction and refinement policies (Knight, 2016) and also to control for inter-litter differences. However, with certain tasks such as fear conditioning this may have been a confounding factor. Perhaps during adolescence the animals robustly acquired the memory and this affected their performance during adulthood. Thus, to validate these findings further, a study using separate cohorts for testing during adolescence and adulthood is warranted. Furthermore, spontaneous alternation in the Y maze, which is a measure of spatial memory was affected in both male and female adolescent mice, but not adults. Since both male and female Nr2e1$^{-/-}$ mice displayed impaired performance, we suggest that this type of behaviour is strongly associated with the deficiency in TLX and the single-housing of the males does not confound the result. However, the performance reached the same levels as wildtype controls by adulthood, which suggests that perhaps compensatory mechanisms take place between adolescence and adulthood that level off the performance.

Due to the large number of counfounding factors within the mouse study (single housing, visual deficiencies and other developmental problems that may have occurred due to the spontaneous deletion), we chose to test the involvement of TLX in cognitive

performance using another method – namely lentiviral delivery of TLX shRNA directly into the DG of adolescent rats. This method allows for more control over what brain areas and cells are affected and what phenotype we expect to see. By adolescence, TLX is only present in the neurogenic niches and hence cannot affect the neurons that are already mature and established within the circuitry (Monaghan et al., 1995). To the best of our knowledge, our lentiviral approach was the first study to examine the effect of TLX knockdown at the start of adolescence on cognitive performance during two different periods of the lifespan. Interestingly, we did not observe any deficits in learning and memory in response to TLX knockdown either during adolescence, or adulthood. We observed a decrease in the proliferating cells in the DG in the rats that received shRNA treatment, however, the relative decrease was small, perhaps insufficient to incur a disruption in learning and memory performance. Another explanation of our findings would be that our model did not target immature neurons, but rather their precursors and progenitors. However, evidence shows that it is the newborn neurons which are implicated in tasks such as pattern speration and spatial memory (Aimone et al., 2011; Clelland et al., 2009; Leutgeb and Leutgeb, 2007). Thus, perhaps our intervention on the regulation of NS/PCs is not severe enough to affect the number and function of newly produced neurons and to therefore result in impaired learning and memory. Additionally, the adolescent cohort began behavioural testing only two weeks after the lentiviral delivery. Even though this is sufficient time for the virus to functionally integrate and produce the desired knockdown effects (Carter and Shieh, 2015; Crespo-Barreda et al., 2016; Kher et al., 2011), it is not sufficient time to perhaps influence the newborn neuronal outcome. Newborn cells take up to 4 weeks to mature and up to 6-7 weeks to integrate in

circuitry (Kempermann et al., 2015). Adolescence in rodents only lasts about 4 weeks thus a different approach, one that would produce a more rapid genetic knockdown, of TLX depletion could be used to see the precise effects in this time period. Furthermore, with respect to the adult cohort, it is possible that by depriving the hippocampal NSCs of TLX expression during early adolescence, the cells developed an adaptive strategy to overcome the lack of TLX and thus cognitive performance measured 3 months post lentiviral administration had reverted to baseline levels. As discussed, adolescence is a critical period of heightened plasticity, thus it is possible that damage to one pathway in this moment of development was rescued by enhancement of a parallel pathway with a similar function. Additionally, it is possible that deficits in memory and learning would be observed at an intermittent period in early adulthood, when perhaps compensatory mechanisms have not yet been fully implemented. Lastly, it would be very interesting to compare interventions where on one hand the hippocampal NSC pool is depleted, while on the other hand newborn neurons are inhibited from integration in circuitry, and to determine which intervention has a more deleterious effect (if either does) on memory and learning performance. Importantly, the adolescent cohort of rats that underwent TLX knockdown exhibited a hyperactive phenotype, similar to the one observed in the mice with spontaneous deletion for TLX, a phenomenon that did not persist into adulthood. To confirm that this consistency is directly linked to the TLX deficiency, a future study would focus on investigating the changes in connectivity between the striatum and hippocampus that may occur as a result of the absence of the nuclear receptor.

7.5 Conclusion

Is TLX a valid therapeutic target? Our molecular results suggest that it is. If TLX is in direct communication with the immune cells of the brain (as it appears by association from Chapter 2 & 3), then compounds affecting this interaction could be used when there is pathological activation of microglia for instance. Additionally, pharmacological manipulation of the TLX gene could promote endogenous NSCs differentiation into neurons and astrocytes, which could be a useful tool for neurodegenerative conditions, where neuronal loss needs to be tackled. What is more, due to its specific location within the CNS, a small molecule targeting TLX is unlikely to have impact on other tissues to cause potential side effects (Benod et al., 2016). Whether our behavioural data, which shows mixed findings relating to the validity of TLX in treatment of disorders affecting cognition, are due to the different models and species used remains to be illuminated. Gene-environment interactions may also have cumulative effects that would only be apparent in multi-hit models. Taken together, we propose that TLX is an important regulator of NSCs maintenance and that this biological process has huge potential in repopulating areas of injury or trauma, as well as areas affected by pathological degeneration.

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Appendix A

Lentiviral Packaging and titration

Introduction

Lentiviral vectors integrate genes into the host-cell chromosome and thereby cause a long-term expression or silencing of target genes. They can penetrate the nucleus of non-dividing cells, making them ideal to target neurons. The feline immunodeficiency viral (FIV) backbone is a non-primate-based vector, which has been preferred over the HIV-based vector for safety reasons. The wildtype FIV has a tropism for cats and there have been no reports to date of human infection. It has been shown *in vitro* that the efficacy of FIV vectors is maintained over time and even can be longer-lasting than that of adenoviral vectors.

Method

Plasmids were purchased from System Bioscience in the form of bacterial streaks carrying custom cDNA constructs for TLX overexpression and a control GFP-loaded construct. For the control plasmid pCDF-CMV-IRES-GFP (Cat# 140128-3; see Figure A.1), IRES-GFP was cloned into CD100A-1 and the clone was verified by CMV forward, IRES reverse and WPRE reverse sequencing primer. The construct carrying the target gene, pCDF-CMV-mNr2e1-IRES-GFP (Cat#140128-4; See Figure A.2) mouse Nr2e1 was cloned into pCDF-CMV-IRES-GFP promoter and the clone was verified by CMV forward and IRES reverse sequencing primer.

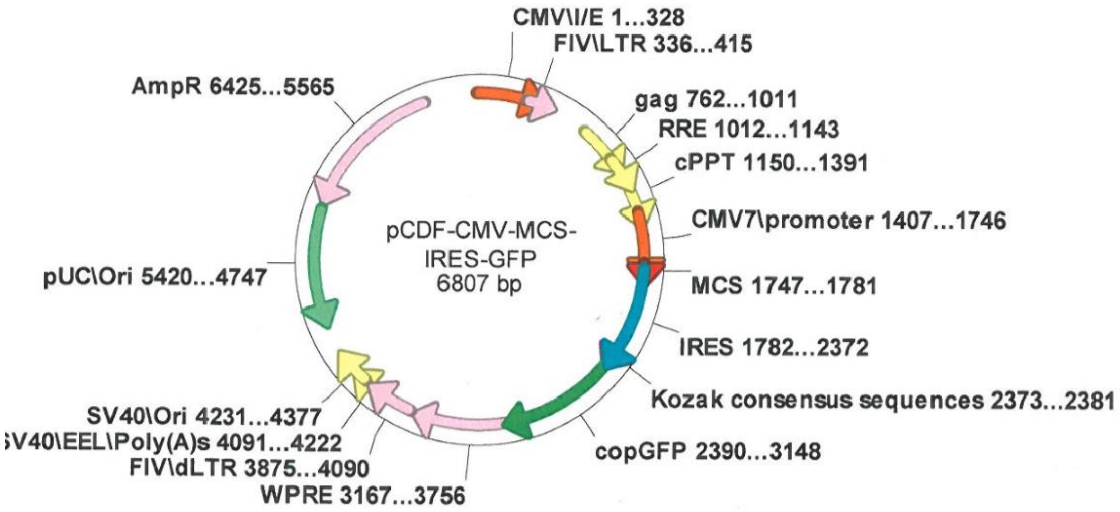

Figure A.1: Control plasmid

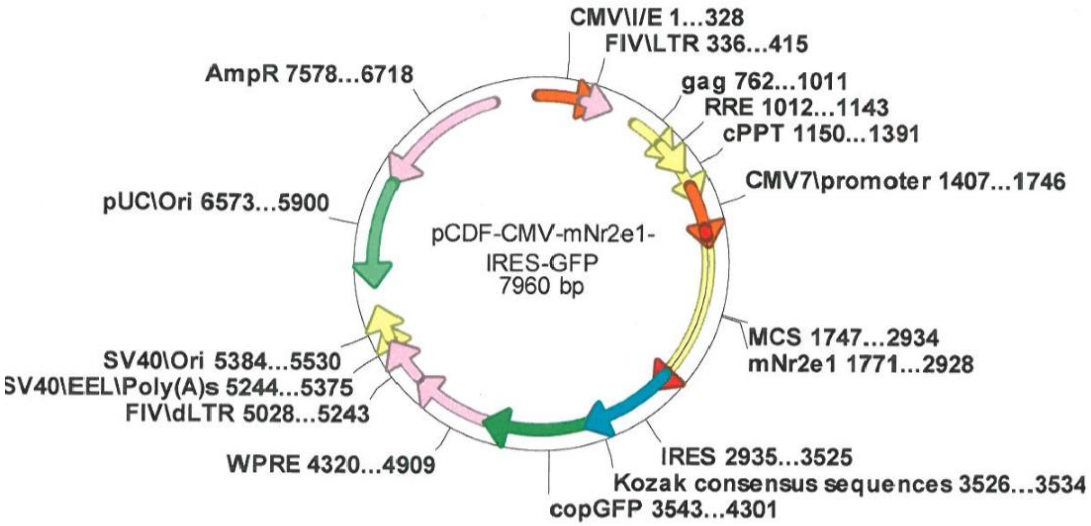

Figure A.2: Plasmid overexpressing TLX

Plasmids were purified using GenElute HP Endotoxin-Free Plasmid Maxiprep Kit (Cat# NA0410, Sigma) according to the manufacturer's instructions. Briefly, bacteria were harvested and incubated overnight. The pellet was collected after 10-minute centrifugation at 5000 x g and was resuspended in 12 ml of Resuspension/RNase

Solution. Subsequently, bacteria were lysed and lysate was neutralized with chilled Neutralization Solution. The lysate was then filtered and transferred to a binding column. Two washes were applied to remove contaminants and the purified plasmid DNA was eluted with endotoxin-free water.

The lentiviral particles were assembled using three separate plasmids containing the pPACKF1 Lentivector Packaging Kit (Cat# 140919-001, System Bioscience) according to manufacturer's instructions. Briefly, 293TN producer cells were co-transfected with the lentiviral expression construct of interest (GFP-expressing control plasmid or target gene (TLX/GFP)-expressing plasmid) and the pPACK packaging plasmid mix with Lipofectamine Reagent (Cat# LV900A-1, SBI) and Plus Reagent (Invitrogen) in a 15-cm culture plate. The pPACKF1 is a mixture of FIV lentiviral packaging plasmid (pFIV-34N) and pVSV-G plasmid. These constructs provide all the necessary structural, regulatory, and replication proteins required to produce VSV-G pseudotyped lentiviral particles when co-transfected with an FIV-based lentiviral expression construct into a producer 293TN cell line. Pseudotyped viral particles with VSV-G envelope protein can infect a variety of both mammalian and non-mammalian cells. Viral particles were collected after 48 hours and stored at -80 for titer determination at a later stage.

In order to determine the viability of the packaged lentiviral particles, HEK293 target cells were infected with GFP-expressing and TLX-expressing viral particles. After

growing the cells in the presence of the lentiviral constructs for 72 hours, the percentage of target cells that were transduced with pseudoviral stock was assayed using FACS and titer was calculated.

Results

The successful transfection of the producer cell line was achieved with both constructs as illustrated by the example images below.

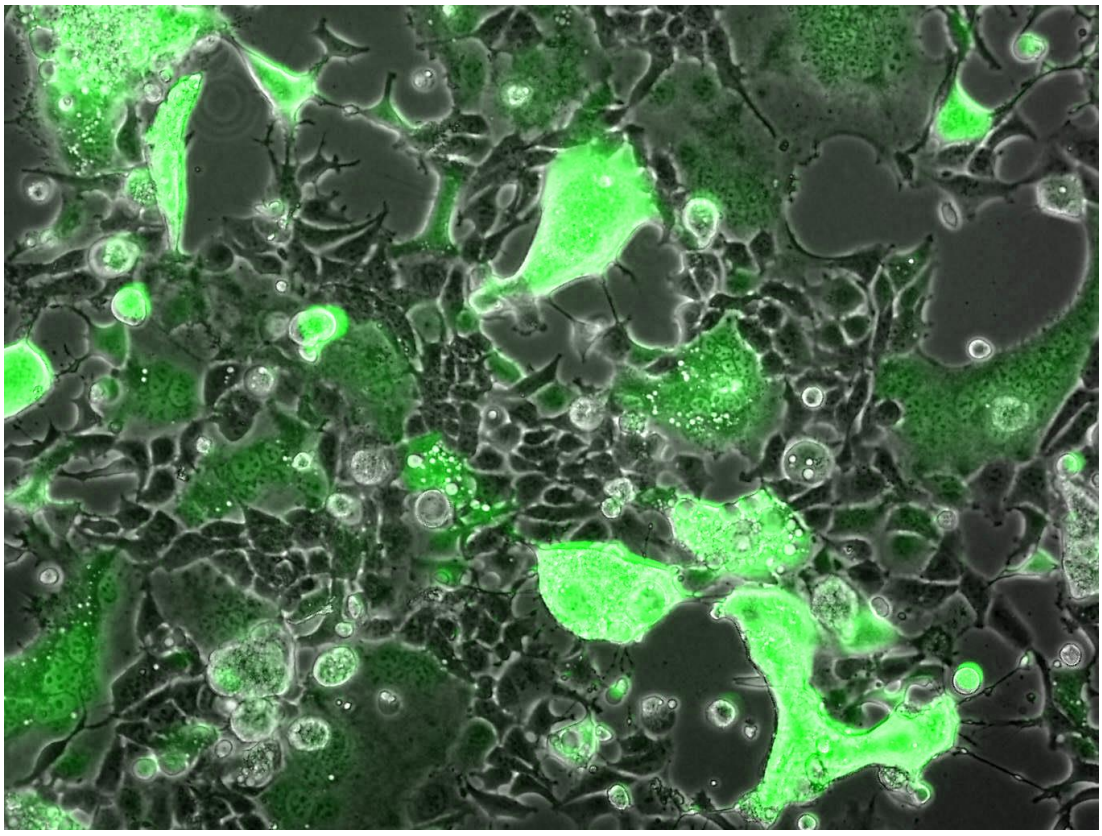

Figure A.3: 48 hours after 293TN cells have been transfected with GFP-expressing plasmid and pPACKF1 packaging plasmid mix.

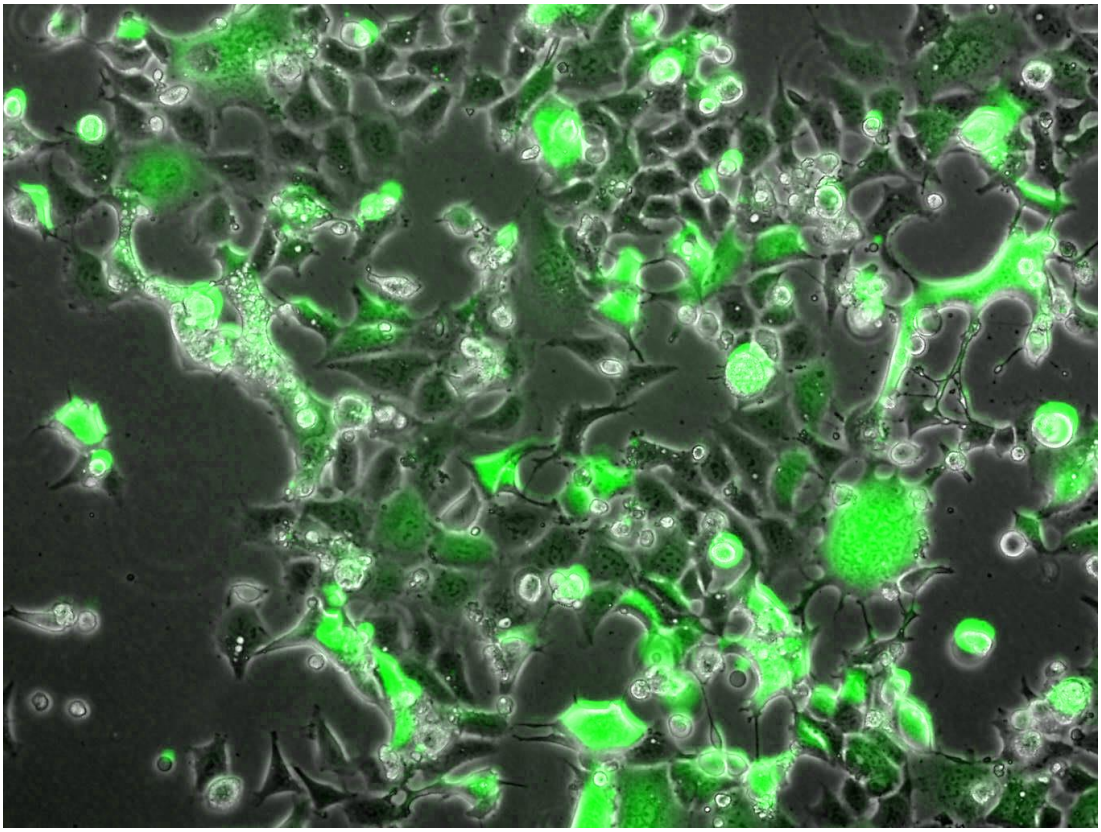

Figure A.4: 48 hours after 293TN cells have been transfected with TLX-expressing plasmid and pPACKF1 packaging plasmid mix.

After transformation, the viral titer was ~ $1x10^6$ TU/ml as determined by FACS analysis of the HEK293 cells infected by the pseudoviral supernatant. Example images below of the transduced cells show the high infection rate achieved by both lentiviral vectors.

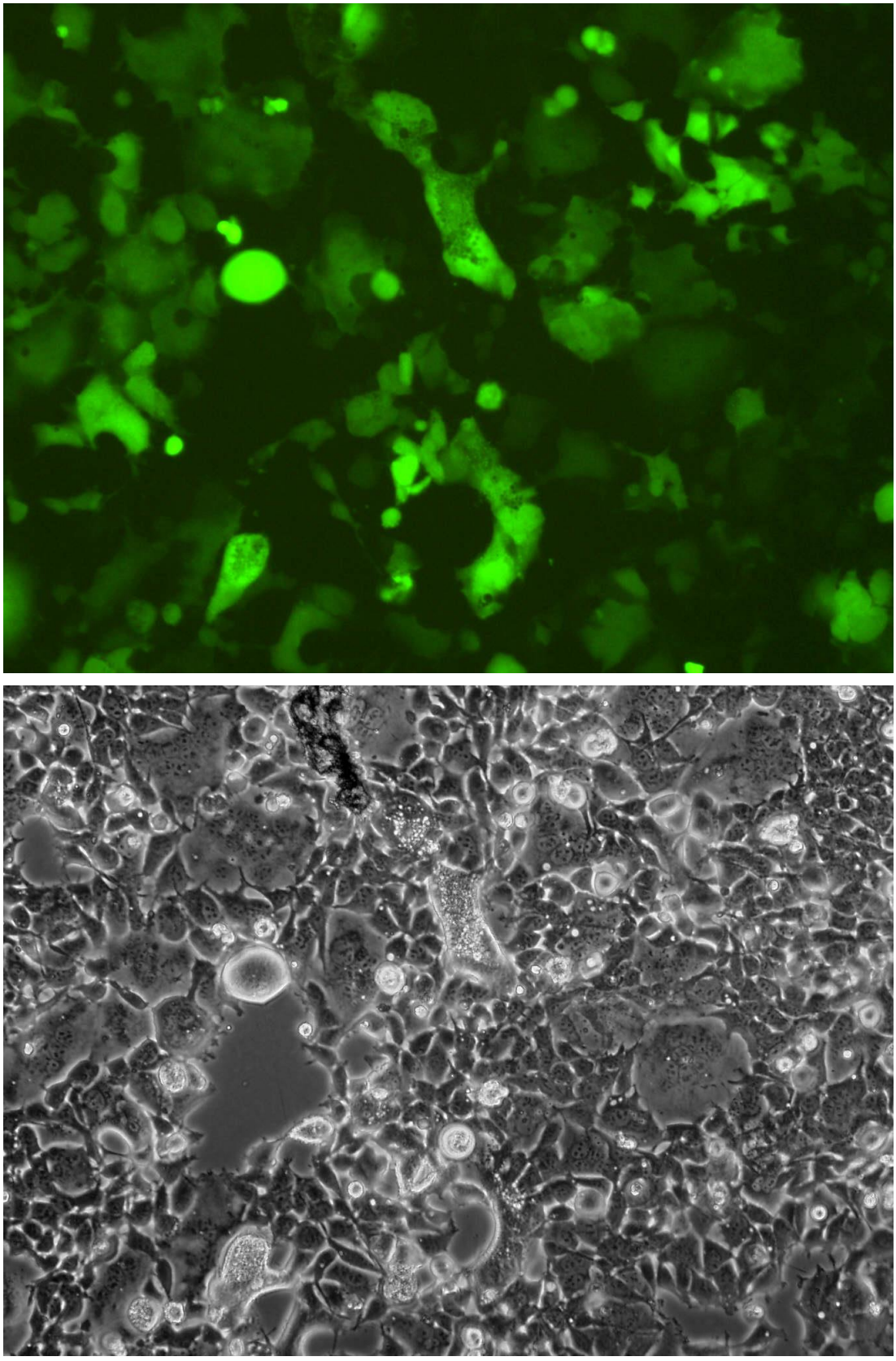

Figure A.5: Fluorescent (top) and a bright field (bottom) images showing HEK293 cells 72 hours post infection with the GFP-expressing pseudovirus supernatant.

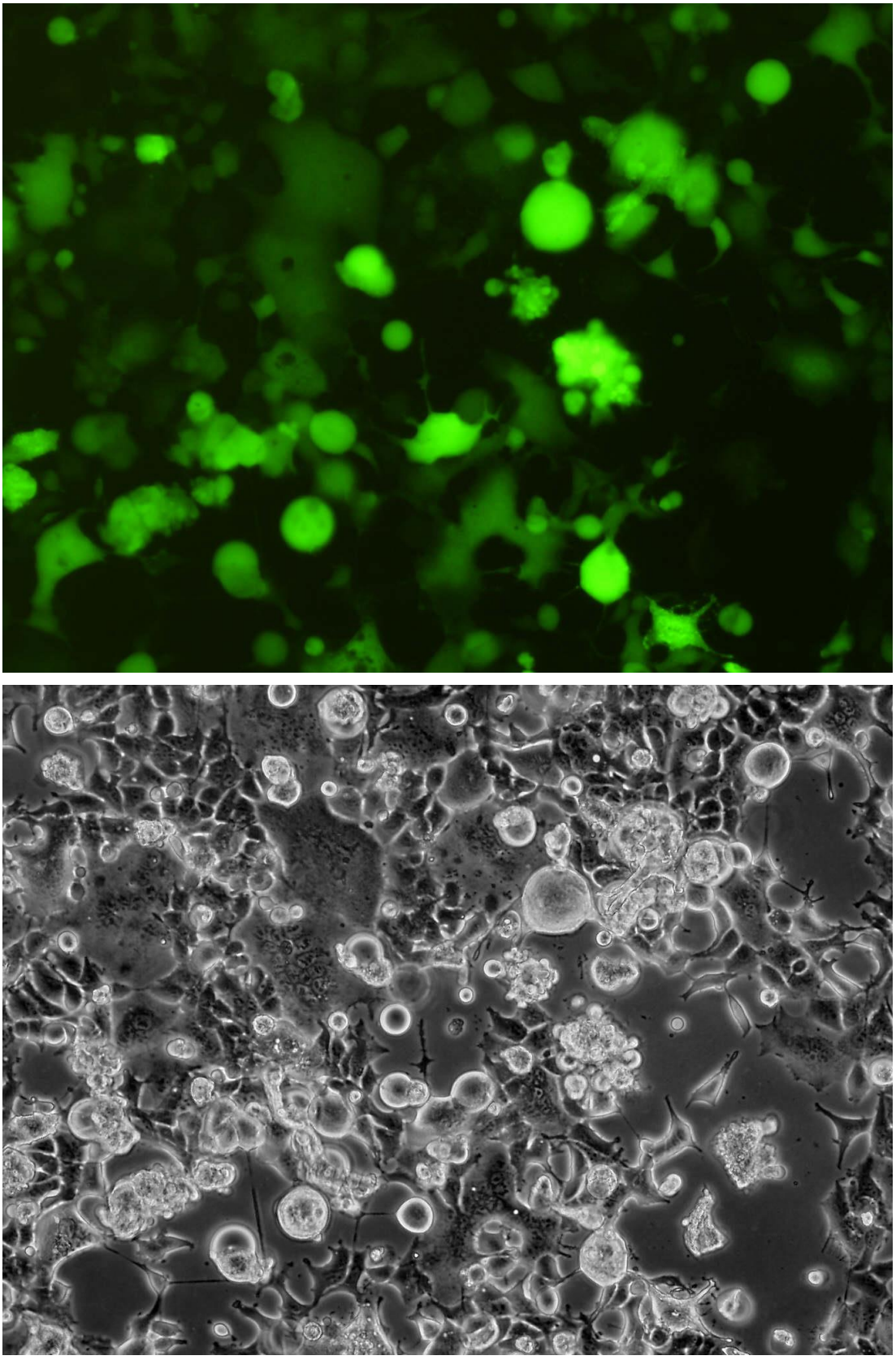

Figure A.6: Fluorescent (top) and a bright field (bottom) images showing HEK293 cells 72 hours post infection with the TLX-expressing pseudovirus supernatant.

Appendix B

Publications & Submissions

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Absence of the neurogenesis-dependent nuclear receptor TLX induces inflammation in the hippocampus

Danka A. Kozareva[a], Cara M. Hueston[a], Ciarán S. Ó'Léime[a], Suzanne Crotty[a], Peter Dockery[b], John F. Cryan[a,c], Yvonne M. Nolan[a,c,*]

[a] Department of Anatomy and Neuroscience, University College Cork, Ireland
[b] Department of Anatomy, National University of Ireland, Galway, Ireland
[c] APC Microbiome Institute, University College Cork, Ireland

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ABSTRACT

The orphan nuclear receptor TLX (Nr2e1) is a key regulator of hippocampal neurogenesis. Impaired adult hippocampal neurogenesis has been reported in neurodegenerative and psychiatric conditions including dementia and stress-related depression. Neuroinflammation is also implicated in the neuropathology of these disorders, and has been shown to negatively affect hippocampal neurogenesis. To investigate a role for TLX in hippocampal neuroinflammation, we assessed microglial activation in the hippocampus of mice with a spontaneous deletion of TLX. Results from our study suggest that a lack of TLX is implicated in deregulation of microglial phenotype and that consequently, the survival and function of newborn cells in the hippocampus is impaired. TLX may be an important target in understanding inflammatory-associated impairments in neurogenesis.

1. Introduction

The process of generating functional neurons from stem and progenitor cells in the central nervous system occurs in the adult as well as in the embryonic brain. Neurogenic niches have been identified in the adult mammalian brain including the subgranular zone (SGZ) of the hippocampus. Here, the progeny of SGZ stem cells migrate to the granule cell layer (GCL) of the dentate gyrus (DG) and integrate into hippocampal circuitry as mature excitatory neurons (Gage, 2000). Adult hippocampal neurogenesis has been implicated in learning and memory (Gould et al., 1999; Kempermann, 2008) and has been shown to play a role in mood regulation (Balu and Lucki, 2009; O'Leary and Cryan, 2014). Moreover, decreased hippocampal neurogenesis is recognized as an important mechanism underlying cognitive deficits associated with depression as well as with normal aging (Kuhn et al., 1996; Lazarov and Marr, 2010; Marlatt and Lucassen, 2010; Spalding et al., 2013). The precise mechanisms underlying the impairment of hippocampal neurogenesis and associated cognitive impairment are not yet fully understood, however, a role for a number of intrinsic factors have been proposed (Qu and Shi, 2009; Green and Nolan, 2014; O'Leary et al., 2016b).

One such intrinsic factor is the orphan nuclear receptor subfamily 2 group E member 1 (NR2E1), also known as TLX. The alignment of the TLX gene is highly conserved across species (97% homology between mouse and human; Jackson et al., 1998). Expression of TLX is restricted to the neurogenic niches of the brain as well as the retina (Monaghan et al., 1997; Shi et al., 2004) where it has been shown to be crucial for neural and retinal development (Miyawaki et al., 2004; Li et al., 2008). Specifically, TLX is responsible for the timing of neurogenesis by regulating the proliferation, differentiation and migration of stem cells (Roy et al., 2004) and has been termed a master regulator of neurogenesis (Islam and Zhang, 2015). Moreover, targeted disruption and conditional deletion of TLX in mouse models have implicated TLX as an important factor in the control and maintenance of adult hippocampal neurogenesis (Roy et al., 2004; Niu et al., 2011; Murai et al., 2014), and in hippocampal neurogenesis-associated behavioural tasks in rodents such as Morris Water Maze and contextual fear conditioning (Zhang et al., 2008; Murai et al., 2014). In a spontaneous deletion mouse model, adult TLX knock out mice displayed altered neurogenesis, synaptic plasticity, and an impairment of dendritic structures in the DG.

Abbreviations: ANOVA, one-way analysis of variance; Arg1, Arginase1; BrdU, 5-bromo-2′-deoxyuridine; CD206, C-Type Mannose Receptor 1; DCX, doublecortin; DG, dentate gyrus; GFAP, glial fibrillary acidic protein; GCL, granule cell layer; H & E, haemotoxylin and eosin; Iba-1, ionized calcium binding adaptor molecule 1; IL-1β, interleukin-1 beta; i.p., intraperitoneal; NDS, normal donkey serum; Nr2e1/TLX, tailless homolog orphan nuclear receptor subfamily 2 group E member 1; NSC, neural stem cellPBS, phosphate buffered saline; PFA, paraformaldehyde; SGZ, subgranular zone; SVZ, subventricular zone; TNFα, tumor necrosis factor alpha

* Corresponding author at: Department of Anatomy and Neuroscience, University College Cork, Ireland.
E-mail address: y.nolan@ucc.ie (Y.M. Nolan).

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These mice also presented with impairments in motor, cognitive and anxiety-related behaviours (Young et al., 2002; Christie et al., 2006). Mice with constitutive knock out of the TLX gene present with lower body weight, hypoplasia and distortion of the anterior aspects of the brain. In addition, the Nr2e1$^{-/-}$ mice exhibited thin and frail rostral commissure, the cerebrum and olfactory lobes were underdeveloped and the retina suffered diminished vascularization and displacement of retinal ganglion cells and optic nerves (Young et al., 2002).

Microglial cells and pro-inflammatory cytokines in the microenvironment of the subgranular neurogenic niche have also been shown to impact upon neurogenesis (Yirmiya and Goshen, 2011; Green and Nolan, 2014). Microglia are the resident immune cells of the central nervous system and have been shown to regulate adult hippocampal neurogenesis under physiological conditions (Sierra et al., 2014). Quiescent (also known as ramified or resting) microglia display small cell bodies and multiple extending processes, with which they probe the environment for signals of injury, trauma or other disruption of homeostasis. However, under inflammatory conditions, microglia become activated and can suppress neurogenesis (Belarbi and Rosi, 2013). Activated microglia become motile using amoeboid-like movements and display enlarged cell soma and one or less extending process (Kettenmann and Verkhratsky, 2011; Kohman and Rhodes, 2013). Activated microglia have been described as either adopting a pro- (M1) or an alternatively activated-(M2) inflammatory phenotype (Orihuela et al., 2016). Pro-inflammatory or M1 microglia are phagocytic and release inflammatory molecules including cytokines, nitric oxide and other reactive oxygen species which have been shown to have a negative effect on hippocampal neurogenesis (Ekdahl et al., 2003; Fujioka and Akema, 2010; Belarbi et al., 2012). Two such cytokines shown to be released by activated microglia are interleukin − 1 beta (IL-1β) and tumor necrosis factor alpha (TNFα) (Nakamura et al., 1999; Wang et al., 2015). We have previously shown that IL-1β treatment decreases TLX expression in neurosphere cultures prepared from both embryonic and adult rat hippocampus in a time- and dose- dependent manner (Green and Nolan, 2012; Ryan et al., 2013). Alternatively activated (M2) microglia, on the other hand, have been shown to exhibit neuroprotective properties (Orihuela et al., 2016) by releasing growth factors, enzymes and cytokines which facilitate repair and neurite outgrowth (Butovsky et al., 2006; Cherry et al., 2014). For instance, the enzyme Arginase 1 (Arg1) is released in response to wound healing and extracellular matrix deposition (Cherry et al., 2014), while the expression of C-Type Mannose Receptor 1 (CD206) is promoted in response to stimulation by the anti-inflammatory cytokine IL-4 (Roszer, 2015). It should be noted however that it has been suggested that describing activated microglia as M1 or M2 type can be restrictive and can hinder our understanding of the complex pathways *via* which microglia influence the brain parenchyma (Ransohoff, 2016).

The role of TLX in the activation status of microglia in the hippocampal neurogenic niche remains largely unexplored. Thus, the aim of the present study was to investigate microglial phenotypes and the hippocampal architecture in heterozygous and homozygous mice with spontaneous deletion of TLX.

2. Material and methods

2.1. Animals

Two month old male Nr2e1$^{-/-}$ knock-out mice, Nr2e1$^{+/-}$ heterozygous mice, and wildtype controls (129S1/SvImJ background) were group housed under standard housing conditions (temperature 21 °C and relative humidity 55%), with food and water available *ad libitum*. Breeding pairs were kindly provided by Prof. Elizabeth Simpson, University of British Colombia. Nr2e1$^{-/-}$ mice exhibit a spontaneous deletion of the entire TLX allele, including all nine exons. However, the deletion of TLX does not affect the transcription of neighbouring genes (Kumar et al., 2004). All experiments were conducted in accordance with the European Directive 2010/63/EU, and under an authorization issued by the Health Products Regulatory Authority Ireland and approved by the Animal Ethics Committee of University College Cork.

2.2. BrdU administration and tissue preparation

Bromodeoxyuridine (BrdU; Sigma) was administered (4 x intraperitoneal (i.p.) injections over the course of 6 h at 75 μg/10 mL/kg) to one cohort of Nr2e1$^{-/-}$, Nr2e1$^{+/-}$ and wildtype mice at postnatal day (P) 42. Since the effect of silencing and overexpressing TLX on neuronal survival has been thoroughly examined at 3- and 4-week time points (Roy et al., 2004; Niu et al., 2011; Murai et al., 2014), we investigated the effect of TLX knock out on neuronal and cellular survival 2 weeks post BrdU injection. This allowed us to examine whether a lack of TLX interferes at an intermittent point (2 weeks) of the neurogenic process, rather than when adult-born neurons integrate into hippocampal circuitry (4 weeks). At P56 mice were euthanized with an i.p. injection of anaesthetic (1.0 mL/kg) and transcardially perfused using a 0.9% phosphate buffered saline (PBS) solution followed by 4.0% paraformaldehyde (PFA) in PBS. After overnight incubation in PFA, brains were incubated in 30% sucrose until they sank, and subsequently flash frozen using liquid nitrogen. Coronal sections (40 μm) through the hippocampus were collected directly onto slides in a 1:6 series, then stored at − 80 °C.

2.3. Immunohistochemistry

To determine the survival of adult-born hippocampal neurons, sections were double-labelled with BrdU and the neuronal marker NeuN. DNA was denatured in sections by incubation in 2 M HCl for 45 min at 37 °C, renatured in 0.1 M sodium tetraborate (pH 8.5) and then blocked in 3% normal donkey serum (NDS; Sigma D9663). Slides were incubated with rat anti-BrdU antibody (Abcam AB6326; 1:250) followed by AlexaFluor594 donkey anti-rat (Abcam Cat# ab150156; 1:500) and mouse anti-NeuN (Millipore MAB377; 1:100) antibodies. Finally, sections were incubated with AlexaFluor488 donkey anti-mouse (Abcam Cat# ab150105; 1:500) antibody, washed and coverslipped using anti-fade mounting medium (Dako; Cat# S3023).

The number of microglia and newly born neurons in the hippocampus was assessed by staining for ionized calcium binding adaptor molecule 1 (Iba-1) and doublecortin (DCX), respectively. To determine whether TLX was expressed on microglia, double-labelling was performed with TLX and Iba-1. Astrocytic activation was assessed using an antibody raised against the glial fibrillary acidic protein (GFAP). Sections were washed, incubated in 3% NDS and then in anti- Iba-1 (Wako 019-19741; 1:1000) or anti-DCX (Santa Cruz Biotechnology; Z0334; 1:100), the combination of TLX (Abcam ab86276; 1:100) and Iba1 (Abcam ab5076; 1:100), or in GFAP (DAKO z0334; 1:500) antibodies. Sections were incubated in AlexaFluor488 donkey anti-rabbit (GFAP and Iba-1; Abcam ab150073; 1:500) or AlexaFluor488 donkey anti-goat (DCX; Abcam ab150129; 1:500) or the combination of AlexaFluor488 donkey anti-rabbit (TLX; Abcam ab150073; 1:500) and Alexa Fluor594 donkey anti-goat (Iba1; Abcam ab150136; 1:500) antibodies and were subsequently counterstained with DAPI (Sigma D9642; 1:5000), and coverslipped using anti-fade mounting medium (Dako; S3023).

For haemotoxylin and eosin (H & E) staining, slides were washed in distilled water, incubated in haemotoxylin, washed and incubated in eosin. Slides were dehydrated in a series of alcohol concentrations 70%, 90%, 95% and 100%, followed by incubations in histolene.

2.4. Image analysis and cell quantification

Images were obtained using either an Olympus VS120 virtual slide scanning system (courtesy of Prof. Peter Dockery, Department of

Anatomy, NUI Galway, Ireland) or an Olympus FV1000 scanning laser confocal system (BioSciences Imaging Centre, Department of Anatomy and Neuroscience, University College Cork, Ireland). *Z*-stack images with a 1.10 µm or 4.4 µm step size were collected using a 10 × objective (BrdU/NeuN), 20 × objective (DCX/DAPI; Iba1/DAPI) or 40 × objective (GFAP). The DG was imaged bilaterally on all sections. For bright field images an Olympus BX533 upright microscope coupled to an Olympus DP72 camera was used with 10 × and 40 × objectives.

Cell quantification, area and volume measurements were performed using the image processing software packages OlyVIA and ImageJ (National Institute of Health, USA; Schneider et al., 2012). Quantification of BrdU- and BrdU/NeuN- positive cells was performed for the GCL, SGZ, and the hilus of the DG. Quantification of Iba-1-positive cells was performed for the GCL and hilus of the DG. The DCX-expressing cell bodies only emerge in the SGZ and were thus quantified within this area. Systematic random sampling was employed for all cell quantifications, and the unbiased Physical Paired Dissector method (Mayhew, 1992) was performed for unbiased stereological estimation of the number of each immunopositive cell phenotype (Crotty et al., 2008). For area quantification, the area of interest, *i.e.* subareas of the DG or the microglia soma, was outlined manually and its area calculated using the image processing software ImageJ. For analysis of the microglial phenotypes, Iba1 + cells with soma area equal to or below one standard deviation above the mean and more than two visible processes were categorized as ramified or resting, while cells with enlarged soma area (greater than one standard deviation above the mean) and up to one extending process were categorized as activated. The morphology of DCX-positive cells was analysed with NeuronJ, an ImageJ plug-in which facilitates the tracing and quantification of elongated image structures (Meijering et al., 2004; Meijering, 2010). Specifically, the dendritic length, number of dendrites and number of nodes for 10 randomly selected cells through the DG from each of five animals per group were compared across all three groups.

2.5. Quantitative RT-PCR analysis of hippocampal tissue

Fresh brains from wildtype, Nr2e1$^{+/-}$, and Nr2e1$^{-/-}$ were snap frozen, and stored at − 80 °C. The hippocampal region was micropunched according to visual comparison to the mouse brain atlas (Franklin, 2008).

Samples were processed according to the GenElute kit protocol (Sigma; RTN350). Briefly, total cellular RNA was homogenized into lysis solution and homogenized sample was filtered through a binding column to remove non-RNA from the sample. Equal volume of 70% ethanol was added to the filtrate and purified through columns, which were then washed with buffer. Purified mRNA was recovered into 30 µL of elution solution. A further DNAse wipeout step was conducted on the sample using DNase1 (Sigma; AMPD1) to ensure the complete removal of endogenous DNA from the samples. Total RNA yield and purity were determined using the Nanodrop System (Thermo Scientific). Synthesis of cDNA was performed using 0.5 µg of normalized total RNA from each sample using ReadyScript cDNA synthesis mix (Sigma; RDRT-25RXN). Probed cDNA amplification was performed in a 20 µL reaction consisting of 10 µL KiCqStart qPCR Ready Mix with ROX (Sigma; Cat# KCQS02), 0.1 µL of each forward and reverse primer (final concentration 250 nM), 1 µL cDNA template, and 8.8 µL RNase-free water. Real-time RT PCR was performed in duplicate in a 96-well plate (Applied Biosystems) and captured in real time using the StepOne Plus System (Applied Biosystems). Relative gene expression was adjusted to the housekeeper gene *Trfc*, and quantified using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Primer sequences were: 5′-CCCAAGTATTCTCAGATATGATTTCAA-3′ (forward) and 5′-AAAGGTATCCCTCCAACCACTC-3′ (reverse) for *Trfc*; 5′-TGCCACCTTTTGACAGTGATG-3′ (forward) and 5′-TGATGTGCTGCTGCGAGATT-3′ (reverse) for *IL-1β*; 5′-AGGCACTCCCCCAAAAGATG-3′ (forward) and 5′-TTGCTACGACGTGGGCTAC-3′ (reverse) for *TNFα*; 5′-TGGGTGGATGCTCACACTG-3′ (forward) and 5′-ACAGGTTGCCCATGCAGATT-3 (reverse) for *Arg1*; and 5′-GTGGGGACCTGGCAAGTATC-3′ (forward) and 5′-CACTGGGGTTCCATCACTCC-3′ (reverse) for *CD206*.

2.6. Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA) with an α-level of 0.05. In cases where data were not normally distributed or the assumption of homogeneity of variance was violated, the non-parametric Kruskal-Wallis analysis of variance by ranks was performed. Posthoc analyses were conducted using either Bonferroni's multiple comparisons or the Mann-Whitney multiple comparisons test for parametric and non-parametric data, respectively. All data are presented as mean ± SEM.

3. Results

3.1. Impaired hippocampal architecture and reduced neurogenesis and survival of adult-born cells in Nr2e1$^{-/-}$ but not Nr2e1$^{+/-}$ mice

Histological examination of coronal sections through the hippocampus of Nr2e1$^{-/-}$ mice identified distortion of the hippocampal structure compared with wildtype and Nr2e1$^{+/-}$ littermate controls (Suppl. material; Fig. A.1). The vulnerable nature of the tissue from Nr2e1$^{-/-}$ mice resulted in the appearance of vacuoles in the tissue. Fluorescent labelling with the nuclear stain DAPI illuminated the gross impairments in the structure of the DG of Nr2e1$^{-/-}$ mice that were also apparent in the H & *E*-stained sections. While no differences were evident between the dentate gyri of mice heterozygous for TLX and their wildtype littermates, the animals lacking TLX exhibited a notably smaller dentate structure with a significantly altered shape. Specifically, the upper and lower blades of the DG were both shorter and the hilus appeared wider in the Nr2e1$^{-/-}$ mice (Suppl. material; Fig. A.1). We observed a significant decrease in the number of surviving adult-born cells (BrdU-positive) in the DG of Nr2e1$^{-/-}$ mice compared to either wildtype or Nr2e1$^{+/-}$ mice ($F(2, 10) = 6.354$; $p < 0.001$). This difference was driven by a reduction of BrdU-positive cells in the SGZ of Nr2e1$^{-/-}$ mice ($F(2, 10) = 5.115$; $p < 0.05$) compared to their wildtype and heterozygous littermates; no significant change was observed across the three genotypes in the GCL ($F(2, 10) = 0.159$; $p > 0.05$) or the hilus ($F(2, 10) = 1.285$; $p > 0.05$; Fig. 1A). There was a significant decrease in the mean number of surviving new neurons in the DG of Nr2e1$^{-/-}$ compared to wildtype mice ($H(2) = 6.12$; $p < 0.05$). Additionally, the number of surviving new neurons in the SGZ was significantly lower in Nr2e1$^{-/-}$ compared to wildtype and heterozygous littermates ($H(2) = 7.71$; $p < 0.05$). There was no significant effect of genotype in either the GCL ($H(2) = 1.19$, $p = 0.55$) or the hilus ($H(2) = 0.08$, $p = 0.96$; Fig. 1B; C–E). There was a significant decrease in the number of DCX-positive cells in Nr2e1$^{-/-}$ mice ($F(2, 9) = 22.65$; $p < 0.001$) compared to both wildtype and Nr2e1$^{+/-}$ animals (Fig. 2A). DCX-positive cells which extended processes through the GCL were evident in wildtype and Nr2e1$^{+/-}$, but not in Nr2e1$^{-/-}$ mice (Fig. 2B–D). Morphological analysis of DCX-positive cells revealed that Nr2e1$^{-/-}$ mice exhibited a significant decrease in dendritic length ($F(2, 27) = 8.525$; $p < 0.01$; Fig. 2E), a reduced number of dendrites ($F(2, 27) = 4.219$; $p < 0.05$; Fig. 2F) and a diminished number of branching points of the dendritic tree ($F(2, 27) = 4.643$; $p < 0.05$; Fig. 2G–H) compared to wildtype control mice.

3.2. Microglia quantification and phenotype characterization

There was a significant increase in number of microglia in the DG and GCL of Nr2e1$^{-/-}$ mice compared to either their wildtype or Nr2e1$^{+/-}$ littermates (DG: $F(2, 9) = 16.43$, $p < 0.001$; GCL: $F(2, 9) = 30.51$, $p < 0.001$). There was no difference in microglia density in the hilus ($F(2, 9) = 1.435$; $p = 0.28$) across the three genotypes

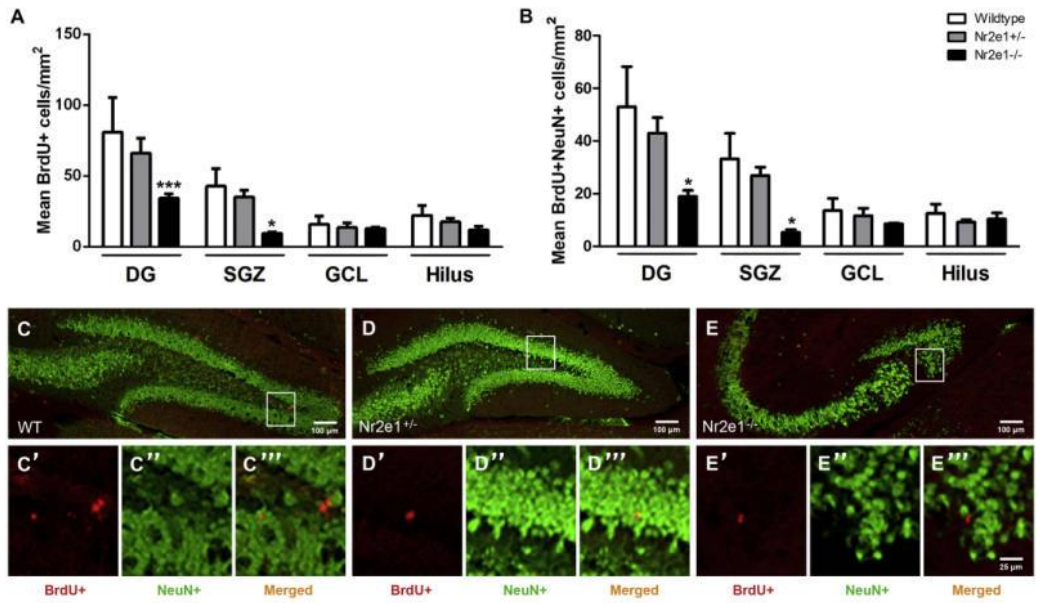

Fig. 1. Lack of TLX causes a reduction in cell survival in the DG of Nr2e1$^{-/-}$ but not Nr2e1$^{+/-}$ mice.
Mean density (cells/mm^2) of adult-born surviving cells (BrdU +; A) and adult-born surviving neurons (BrdU + NeuN +; B) in the DG, SGZ, GCL and hilus of wildtype, Nr2e1$^{+/-}$, and Nr2e1$^{-/-}$ mice. Data are expressed as mean ± SEM. ***$p < 0.001$, *$p < 0.05$ compared to wildtype and Nr2e1$^{+/-}$ mice (ANOVA), n = 4–5. Representative confocal images from wildtype (C), Nr2e1$^{+/-}$ (D), and Nr2e1$^{-/-}$ (E) of coronal sections through the DG immunohistochemically stained with BrdU (red), NeuN (green) and BrdU/NeuN (orange) at 10 × magnification. Scale bar = 100 μm. Higher magnification images depict immunopositive cells in the DG of wildtype (C′: BrdU + channel; C″: NeuN channel; C‴: merged channel), Nr2e1$^{+/-}$ (D′: BrdU + channel; D″: NeuN channel; D‴: merged channel) and Nr2e1$^{-/-}$ (E′: BrdU + channel; E″: NeuN channel; E‴: merged channel). Scale bar = 25 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 3A). Mice lacking TLX displayed microglia with greater cell somal area in the DG and GCL compared to wildtype or Nr2e1$^{+/-}$ mice (DG: $F(2, 9) = 5.387$; $p < 0.05$; GCL: $F(2, 9) = 7.665$; $p = 0.01$; Fig. 3B). Within the hilus we did not observe any differences in microglia size across the three groups ($F(2, 9) = 2.634$; $p = 0.13$; Fig. 3B). The proportion of "resting" or ramified microglia in the DG of mice with complete deletion of the TLX gene was significantly smaller than the proportion of ramified microglia in the wildtype controls ($F(2, 9) = 5.683$; $p < 0.05$). The same pattern was observed in the GCL, but not the hilus (GCL: $F(2, 9) = 5.480$; $p < 0.05$; Hilus: $F(2, 9) = 3.245$; $p = 0.09$; Fig. 3C). Finally, when we compared the proportion of activated microglia (having enlarged soma) across the three genotypes we found a significant increase of activated microglia in Nr2e1$^{-/-}$ mice compared to their wildtype and Nr2e1$^{+/-}$ littermates across all areas examined (DG: $F(2, 9) = 6.531$; $p = 0.01$; GCL: $F(2, 9) = 5.711$; $p < 0.05$; Hilus: $F(2, 9) = 4.119$; $p = 0.05$; Fig. 3D–F). There was a significant increase in the relative mRNA expression of the pro-inflammatory cytokine IL-1β in the hippocampus of mice lacking the TLX gene ($F(2, 15) = 17.79$; $p < 0.001$; Fig. 4A). There was no difference in relative mRNA expression of TNFα across the groups ($F(2, 17) = 0.295$; $p = 0.75$; Fig. 4B). Lastly, when we compared the relative mRNA expression of the alternatively activated markers Arg1 and CD206, we observed no difference across the three genotypes (Arg1: $F(2, 21) = 0.174$; $p > 0.05$; Fig. 4D; CD206: $F(2, 21) = 0.252$; $p > 0.05$; Fig. 4C).

We previously confirmed the expression of TLX in astrocytes derived from cultures of hippocampal neural stem cells (Green and Nolan, 2012). Here we show that immunostaining of hippocampal tissue from wildtype mice revealed no overlap between TLX and Iba-1 expression, (Fig. 5A). Upon examination of GFAP staining, astrocytes appeared to have adopted a reactive or activated phenotype in the hippocampus of Nr2e1$^{-/-}$ mice when compared to Nr2e1$^{+/-}$ and wildtype mice as evidenced by increased fluorescence intensity and hypertrophy of processes (Fig. 5B–D).

4. Discussion

In the present study, we demonstrated a pro-inflammatory phenotype in the DG of Nr2e1$^{-/-}$ mice. Specifically, we observed a significant increase in density of endogenous microglia in the dentate area of Nr2e1$^{-/-}$ mice, while Nr2e1$^{+/-}$ and wildtype mice displayed similar and lower densities of Iba1-positive cells. We found that TLX deficient animals exhibited a higher proportion of microglia that were in an activated state, and reduced percentage of ramified microglia. Furthermore, there was a significant increase in expression of the pro-inflammatory cytokine IL-1β, an indicator of classically activated microglia in the hippocampus of Nr2e1$^{-/-}$ mice, but no difference in the expression levels of TNFα or the alternatively activated markers CD206 and Arg1. These results were coupled with deficits in neuronal morphology and neurogenesis in Nr2e1$^{-/-}$ but not Nr2e1$^{+/-}$ mice as shown by a reduction in the density, dendritic length and nodes of newborn neurons as well as the density of surviving new neuronal and non-neuronal cells in the DG.

The current results support previous work by Monaghan et al. (1997) who showed alteration in the size and architecture of the hippocampus of Nr2e1$^{-/-}$ (in which the gene was functionally knocked down through homologous recombination) compared to wildtype mice (Monaghan et al., 1997). We also showed that the hippocampal architecture of the Nr2e1$^{+/-}$ mice resembles that of wildtype mice. The decrease in the number of adult born neurons in transgenic mice with a

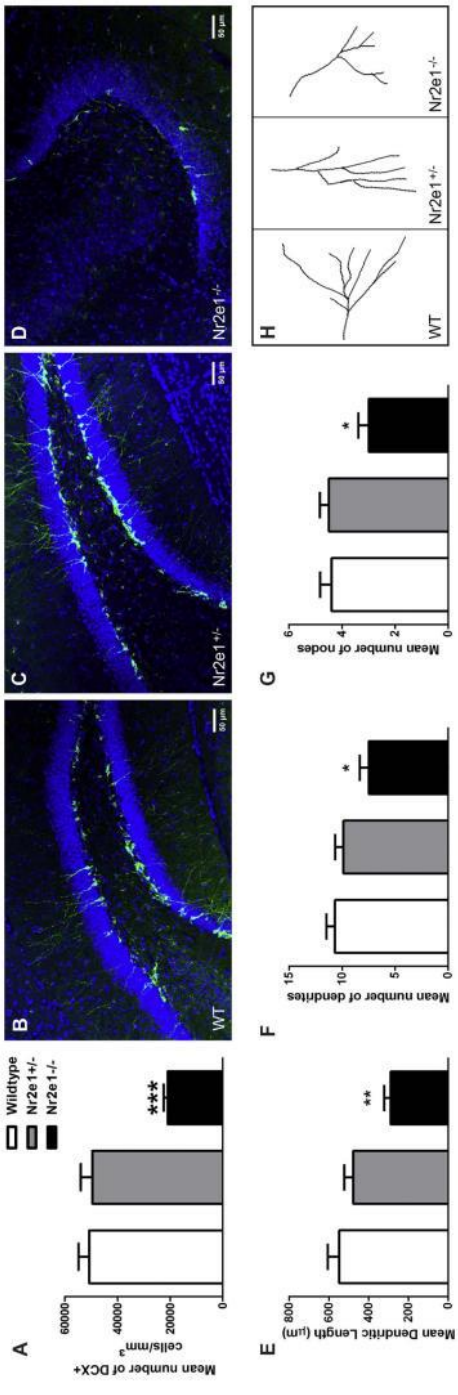

Fig. 2. Lack of TLX is associated with reduction in neurogenesis and impaired morphology of newly born neurons in the DG of $Nr2e1^{-/-}$ but not $Nr2e1^{+/-}$ mice.
Mean number of DCX + cells per mm^3 in wildtype (WT), $Nr2e1^{+/-}$, and $Nr2e1^{-/-}$ animals (A). Representative confocal images through the DG of DCX + (green) cells in wildtype (B), $Nr2e1^{+/-}$ (C), and $Nr2e1^{-/-}$ (D) mice. Nuclei were counterstained with DAPI (blue). Scale bar = 50 µm. Mean dendritic length (µm) of DCX + cells (E), mean number of dendrites per DCX + cell (F) and mean number of nodes per DCX + cell (G) in wildtype, $Nr2e1^{+/-}$, and $Nr2e1^{-/-}$ mice. Data are expressed as mean ± SEM. $^{**}p < 0.001$, $^{*}p < 0.01$, $^{*}p < 0.05$ compared to wildtype and $Nr2e1^{+/-}$mice (ANOVA), $n = 4$–5. Representative tracings of cells from each group (H). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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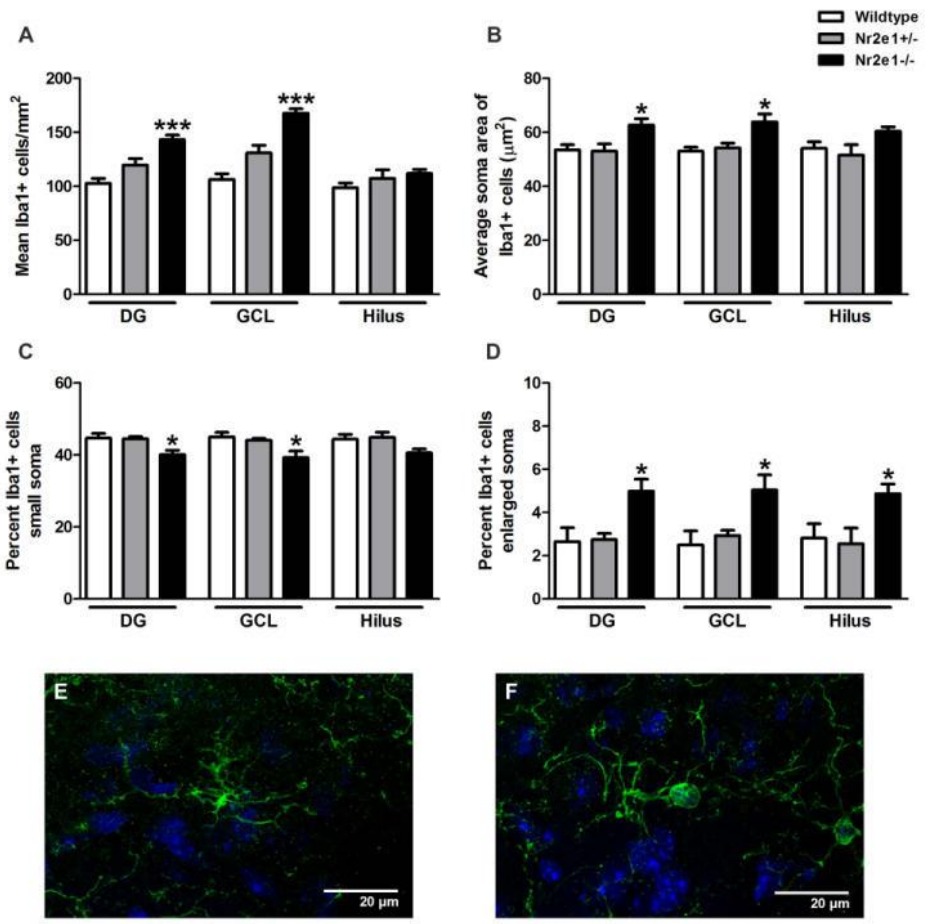

Fig. 3. Lack of TLX is associated with increased microglia density and activation.
The mean density (cells/mm^2) of microglia (Iba1 +) in the DG, GCL and hilus of wildtype (WT), Nr2e1$^{+/-}$, and Nr2e1$^{-/-}$ mice (A). Average microglial soma area (μm^2) in the DG, GCL and hilus of wildtype (WT), Nr2e1$^{+/-}$, and Nr2e1$^{-/-}$ mice (B). Proportion (%) of "resting" (C) and activated (D) microglia in the DG, GCL and hilus of wildtype (WT), Nr2e1$^{+/-}$, and Nr2e1$^{-/-}$ mice. Data are expressed as mean ± SEM. ***$p < 0.001$, *$p < 0.01$ compared to wildtype and Nr2e1$^{+/-}$ mice (ANOVA), $n = 4$. Representative images of a ramified/resting Iba1 + cell (E) and activated Iba1 + cell (F) at 100 × magnification.

targeted deletion of TLX was first described by Shi and colleagues in 2004. Later the Simpson group showed that mice with a spontaneous deletion of TLX not only displayed a decrease in neurogenesis, but also an impairment in synaptic plasticity and in the dendritic structure of pyramidal cells in the DG but not in the CA1 region of the hippocampus (Christie et al., 2006). Here we further demonstrated that mice with a spontaneous deletion of TLX also have reduced survival of newborn neurons as well as newborn non-neuronal cells in the DG as well as impaired morphology of newly born neurons. These findings support previous evidence from studies employing mice with a conditional deletion of TLX that reported that ablation of TLX resulted in a complete loss of transiently amplifying cells and neuroblasts (Roy et al., 2004; Niu et al., 2011). The current results also suggest that a lack of the TLX gene has long term negative consequences for the viability of both neurogenic and gliogenic cells. To the best of our knowledge, we are the first to demonstrate that Nr2e1$^{+/-}$ mice do not exhibit the neurogenic and neuronal survival deficiencies observed in Nr2e1$^{-/-}$ mice. Interestingly, we have previously observed that these Nr2e1$^{+/-}$ heterozygous mice performed similarly to wildtype littermates in contextual fear conditioning, a neurogenesis-associated hippocampal-dependant cognitive task, while Nr2e1$^{-/-}$ mice displayed impaired performance (O'Leary et al., 2016a). Together, these findings suggest that one allele of the TLX gene is sufficient for normal hippocampal neurogenic processes and associated behaviours. This is in line with the proposition that an animal with a heterozygous genotype generally has a higher relative fitness than an animal having a homozygous dominant or homozygous recessive genotype (Charlesworth and Willis, 2009). Indeed, studies have shown, that next to being the master regulator of neural stem cell maintenance (Islam and Zhang, 2015), TLX is also responsible for gliomagenesis in the adult neurogenic niches (Zou et al., 2012). What is more, increased TLX expression in stem cells from gliomas correlated with poor survival of patients (Park et al., 2010),

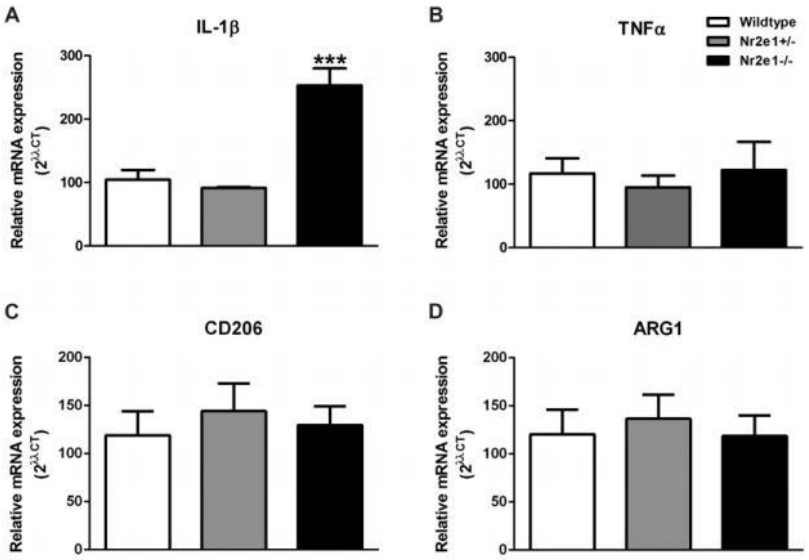

Fig. 4. Lack of TLX is associated with increase in markers of classically but not alternatively activated microglia.
Relative mRNA expression of IL-1β (A) TNFα (B) CD206 (C) and Arg1 (D) in the hippocampus of wildtype, Nr2e1$^{+/-}$ and Nr2e1$^{-/-}$ mice. All values were adjusted to the relative expression of the housekeeping gene *Tyfc*. Data are expressed as the mean ± SEM, ***$p < 0.001$ (ANOVA), $n = 7–9$.

while downregulation of TLX was shown to inhibit tumorigenesis (Xie et al., 2014; Cui et al., 2016). Hence, one allele of the TLX gene may not only be sufficient but also beneficial from an evolutionary perspective.

Interestingly, mice with a spontaneous deletion of TLX did not exhibit full ablation of cell survival and neurogenesis as evidenced by a third of BrdU-positive, BrdU/NeuN-positive and DCX-positive cells remaining in the DG. This could be explained by the fact that neurogenesis is regulated by multiple pathways operating in parallel and/or in synergy that could compensate for the lack of TLX. For example, the Wnt/beta-catenin pathway (Qu et al., 2010), Notch signalling (Breunig et al., 2007; Ables et al., 2010) and the sonic hedgehog (Shh) pathway (Machold et al., 2003; Ahn and Joyner, 2005) have been identified as central mediators of neural stem cell (NSC) maintenance. Indeed, TLX has been shown to directly activate both the Wnt/beta-catenin (Qu et al., 2010) as well as the Notch signalling pathway (Shi, 2015). Conversely, Shh signalling appears to take place upstream of TLX (Shimozaki et al., 2012). It is also possible that in the absence of TLX expression, other transcription factors, such as SOX2 (Shimozaki et al., 2012), autonomously maintain, albeit a reduced pool of NSCs.

Despite the reduction in size of the hippocampus and the reduced cell survival observed, we found a pattern towards an increased number of endogenous microglia in the DG and GCL of the hippocampus of Nr2e1$^{-/-}$ mice. In recent years, microglia have been shown to be important effectors of adult hippocampal neurogenesis. In the absence of inflammation, ramified microglia are involved in pruning newborn cells, in providing trophic support for newly forming cells, and in apoptosis of newborn cells that fail to integrate into the existing circuitry (Sierra et al., 2010; Sierra et al., 2014). *In vitro* studies have also shown that in their resting state, microglia release factors which rescue neuroblasts, instruct neuronal cell differentiation and enhance and prolong the neurogenic potential of the cultured cells (Walton et al., 2006). However, microglia that become activated by foreign antigens or by changes in brain homeostasis are predominantly neurotoxic, promote an inflammatory environment and have been shown to suppress hippocampal neurogenesis by reducing the survival of neuroblasts (Ekdahl et al., 2003; Monje et al., 2003). We thus characterized the activation status of the microglia in the DG of Nr2e1$^{-/-}$ mice by firstly assessing the morphology of the cells, and observed that there was a significant increase in the proportion of activated microglia in the whole DG, including the GCL and hilus, of Nr2e1$^{-/-}$ mice. This result was coupled with a significant decrease in the proportion of ramified Iba1-positive cells in the DG of Nr2e1$^{-/-}$ animals. We showed through immunolabelling that TLX is not expressed on microglia. This finding is corroborated by RNA sequencing data (Zhang et al., 2014) and gene expression analysis and histology (Zou et al., 2012). Future studies should examine whether there is causal link between the lack of TLX expression and the increase in microglial activation that we observed. If such causality exists, it would be important to identify the underlying pathway mediating this process as potential therapeutic targets for inflammatory and/or neurodegenerative conditions may be revealed. We speculate that astrocytes may be key mediator of the activation of microglia under reduced or ablated TLX expression. In our transgenic model, we observed increased intensity of GFAP fluorescent staining and extended or hypertrophic GFAP processes in Nr2e1$^{-/-}$ mice. These parameters have been established as markers of astrocyte activation and reactive gliosis (Wilhelmsson et al., 2006; Pekny et al., 2014; Pekny et al., 2016). Furthermore, it has been shown that TLX regulates astrogenesis in the subventricular zone (SVZ) (Qin et al., 2014) as well as astrocyte development in the retina (Miyawaki et al., 2004). Additionally, loss of TLX expression in the SVZ also resulted in increased GFAP fluorescence staining and extended GFAP-positive cell processes (Li et al., 2012). Whether the spontaneous deletion of TLX in our model causes direct activation of astrocytes, which thereby activate microglia, or whether a lack of TLX results in activation of microglia, which cause astrocytes to adopt reactive phenotype remains to be investigated.

In order to determine whether the increased number of activated microglia in Nr2e1$^{-/-}$ mice display a pro-inflammatory or neuroprotective phenotype, we examined the relative mRNA expression of pro-inflammatory cytokines IL-1β and TNFα as well as the expression of alternatively activated markers Arg1 and CD206 in the hippocampus of

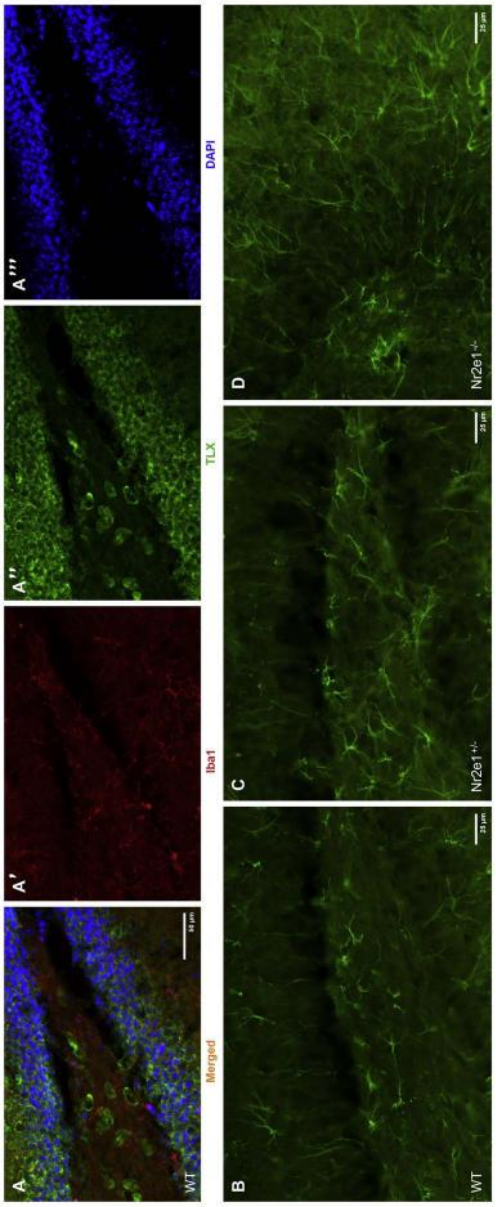

Fig. 5. Lack of TLX expression on microglia and increase in GFAP staining in the absence of TLX.
Representative image of the hippocampus of a wildtype mouse showing lack of colocalizationn between TLX and Iba1 by double immunofluorescence (A). Iba1 + staining appears as red (A'), TLX staining appears as green (A'') and nuclei were counterstained with DAPI (blue; A'''). Scale bar = 50 μm. Fluorescence images of astrocytes stained for GFAP (green) in wildtype (B), Nr2e1 $^{+/-}$ (C), and Nr2e1 $^{-/-}$ (D) mice. Images were taken at 40 × magnification. Scale bar = 25 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

these mice. We found that there was a significant increase in the mRNA expression of IL-1β but not TNFα in Nr2e1$^{-/-}$ compared to wildtype and Nr2e1$^{+/-}$ mice. Furthermore, no difference was observed in the expression of the alternatively activated markers. The appearance of microglial-derived IL-1β under endotoxic conditions was first investigated several decades ago (van Dam et al., 1992). This and a related classic pioneering study implicated IL-1β as a key player in the signalling pathway between neuronal and microglial cells during inflammatory challenge (van Dam et al., 1992; van Dam et al., 1995). Elevated levels of IL-1β have previously been shown to negatively impact upon neurogenesis. For example, central infusion of IL-1β to rats and overexpression of IL-1β in a conditional transgenic mouse model both significantly reduced neurogenesis in the DG (Goshen et al., 2008; Koo and Duman, 2008; Wu et al., 2012). *In vitro* studies have revealed that IL-1β reduced the number of proliferating cells, proliferating newly-born neurons, reduced neuronal differentiation, and stimulated astroglial differentiation in cultures prepared from adult and embryonic rat hippocampal neural precursor cells (Koo and Duman, 2008; Green and Nolan, 2012; Green et al., 2012; Ryan et al., 2013). We have previously demonstrated the vulnerability of TLX to IL-1β in embryonic rat hippocampal NSCs under both proliferation and differentiation conditions (Green and Nolan, 2012). The IL-1β-induced reduction of the numbers of proliferating cultured neural precursor cells and reduction of TLX expression was rescued by inhibition of GSK-3β signalling, which has been shown to negatively regulate neurogenesis (Green and Nolan, 2012). Additionally, we have previously shown that treatment with IL-1β reduced TLX expression in proliferating NSCs in the adult hippocampus in a dose and time-dependant manner. Further, administration of the IL-1 receptor antagonist or IL-1 receptor silencing prevented this decrease (Ryan et al., 2013). Interestingly, others have shown that astrocyte-mediated secretion of IL-1β occurs under inflammatory conditions in human astrocytes cultures (Didier et al., 2003), and spinal cord injury in rats induces increased expression of IL-1β in astrocytes located around the spinal cord ependyma, a neurogenic region (Paniagua-Torija et al., 2015). Recently it was demonstrated that reactive astrocytes are induced by activated microglia (Liddelow et al., 2017), which points to the question of whether activation of astrocytes or microglia occurs first as a result of lack of TLX expression.

Overall, these studies suggest that a lack of TLX expression may be implicated in microglial and astrocytic activation which is coupled with impaired survival and integration of hippocampal newborn neurons. Moreover, increased levels of endogenous IL-1β in the hippocampus of Nr2e1$^{-/-}$ mice correlate with the observed deficiencies in hippocampal neurogenesis and increased microglia activation. Given our previous studies demonstrating that IL-1β induces a decrease in expression of TLX in hippocampal NSCs (Green and Nolan, 2012), it is also likely that a vicious circle of an IL-1β-induced inflammatory environment and an impaired neurogenic phenotype is perpetuated in the absence of TLX expression. Furthermore, it would be important to establish the temporal relation between a lack of TLX expression, astrocyte activation and microglia activation, which may aid in identifying the primary cause for the observed increase in IL-1β in Nr2e1$^{-/-}$ mice.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jneuroim.2017.08.008.

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Research Article

A role for the orphan nuclear receptor TLX in the interaction between neural precursor cells and microglia

Danka A. Kozareva[1,2], Gerard M. Moloney[1], Alan E. Hoban[1], Valerio Rossini[2,3], Ken Nally[2,3], John F. Cryan[1,2] and Yvonne M. Nolan[1,2]

[1]Department of Anatomy and Neuroscience, University College Cork, Cork, Ireland; [2]APC Microbiome Ireland, University College Cork, Cork, Ireland; [3]School of Biochemistry and Cell Biology, University College Cork, Cork, Ireland

Correspondence: Yvonne M. Nolan (y.nolan@ucc.ie)

Microglia are an essential component of the neurogenic niche in the adult hippocampus and are involved in the control of neural precursor cell (NPC) proliferation, differentiation and the survival and integration of newborn neurons in hippocampal circuitry. Microglial and neuronal cross-talk is mediated in part by the chemokine fractalkine/chemokine (C-X3-C motif) ligand 1 (CX3CL1) released from neurons, and its receptor CX3C chemokine receptor 1 (CX3CR1) which is expressed on microglia. A disruption in this pathway has been associated with impaired neurogenesis yet the specific molecular mechanisms by which this interaction occurs remain unclear. The orphan nuclear receptor TLX (Nr2e1; homologue of the Drosophila tailless gene) is a key regulator of hippocampal neurogenesis, and we have shown that in its absence microglia exhibit a pro-inflammatory activation phenotype. However, it is unclear whether a disturbance in CX3CL1/CX3CR1 communication mediates an impairment in TLX-related pathways which may have subsequent effects on neurogenesis. To this end, we assessed miRNA expression of up- and down-stream signalling molecules of TLX in the hippocampus of mice lacking CX3CR1. Our results demonstrate that a lack of CX3CR1 is associated with altered expression of TLX and its downstream targets in the hippocampus without significantly affecting upstream regulators of TLX. Thus, TLX may be a potential participant in neural stem cell (NSC)–microglial cross-talk and may be an important target in understanding inflammatory-associated impairments in neurogenesis.

Introduction

Hippocampal neurogenesis, the process of generating functional new neurons from neural stem cells (NSCs), occurs throughout the lifespan in most mammalian species and plays a role in certain forms of learning, memory and in mood regulation [1]. The discussion on whether hippocampal neurogenesis actually occurs in the adult human brain has recently been renewed [59,60]. However, the hypothesis that adult-generated neurons can make important functional contributions to neural plasticity and cognition across the lifespan in humans is still widely accepted (reviewed in [61]). Microglia are an essential component of the neurogenic niche in the adult hippocampus and provide trophic support for the neurogenic process [2,62]. Specifically, microglia promote the proliferation of neural precursor cells (NPCs) as well as the survival of newly born neurons through the secretion of neurotrophic factors such as insulin-like growth factor 1 (IGF-1) and brain-derived neurotrophic factor (BDNF) [3,4], and play an important role in the pruning of apoptotic adult born neurons immediately after cell birth [5,6].

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Recent evidence shows that microglia in turn can be influenced by neuronal cells [63]. However, the signalling pathways underlying NPC–microglia interaction are yet to be fully explored.

Intracellular cross-talk between neurons and microglia can occur through a variety of signalling mechanisms, one of which is through the chemokine system [7,8]. Signalling occurs between the membrane-bound ligand fractalkine, also known as fractalkine/chemokine (C-X3-C motif) ligand 1 (CX3CL1), which is constitutively expressed by neurons, and its cognate receptor CX3C chemokine receptor 1 (CX3CR1), which in the healthy brain is selectively found on microglia [9–11]. During early postnatal development, signalling between the CX3CL1/CX3CR1 pair drives synaptic pruning [12], elimination of supernumerary neurons [2], and fine-tuning of anatomical connections to ensue correct functional maturation and cell positioning [13,14]. During adulthood, CX3CL1 is expressed at particularly high levels in hippocampal neurons [11] and has been shown to stimulate the survival of NPCs *in vitro* [15]. Direct evidence for the role of CX3CL1/CX3CR1 signalling in adult hippocampal neurogenesis has recently emerged. For instance, targeted knockdown or pharmacological inhibition of CX3CR1 in adult rats resulted in a marked decrease in NPC proliferation and newborn neuron survival in the subgranular zone (SGZ) of the dentate gyrus (DG), the neurogenic niche of the hippocampus [16]. Additionally, it was demonstrated that the CX3CR1-GFP knockout ($CX3CR1^{KO}$) mice and their heterozygote littermates exhibit decreased hippocampal neurogenesis and survival in a gene-dose-dependent manner, which was coupled with reduced synaptic plasticity and impaired performance in neurogenesis-associated tasks, such as contextual fear conditioning and learning in the Morris water maze task [17]. Employing immunohistochemistry and unbiased stereology methods, the authors showed that in the absence of CX3CR1, there was a significant decrease in the number of doublecortin (DCX+) expressing newborn neurons, a marker of neurogenesis. CX3CR1 knockout mice also exhibited reduced proliferation as evidenced by a significantly lower number of cells incorporating the thymidine analogue bromodeoxyuridine (BrdU) at 24 h post injection. Indeed, CX3CR1-deficient mice were found to present with impaired hippocampal neurogenesis, not only during adulthood, but also during late adolescence/early adulthood as well as during aging [18]. Furthermore, using the same model it has been shown that CX3CL1/CX3CR1 signalling is involved in adult hippocampal, but not olfactory bulb neurogenesis [19]. Corroborating these results, another study demonstrated that the decrease in hippocampal neurogenesis in the absence of CX3CR1 expression, was coupled with reduced dendritic complexity and delayed maturation of the newborn neurons [20]. This finding illustrates that CX3CR1/CX3CL1 has a role to play in the dendritic development of new neurons and thus in integration into the neuronal circuitry. It is thus important to recognise that a lack of CX3CR1 in the adult hippocampus has detrimental effects on multiple stages of the neurogenic process from proliferation to survival and morphological maturation [17,20,53]. It has also been shown that the reduced number of DCX+ cells observed in the SGZ of $CX3CR1^{KO}$ mice was specific to knockout of the receptor, as mice that exhibited CX3CL1 knockout did not produce the same deficit [53]. Collectively, these data position the CX3CL1/CX3CR1 pathway as a major mediator of hippocampal neurogenesis.

The orphan nuclear receptor TLX (Nr2e1) whose expression is confined to stem cells in the neurogenic niches of the adult brain is a key intrinsic regulator of hippocampal neurogenesis [21] and it exercises this role by regulating a number of different genes and pathways. For instance, through its action as a transcriptional repressor, it regulates the expression of genes involved in multiple pathways important for the generation of neurons such as cell adhesion [22], DNA replication [23] and cell cycle [24]. It targets genes such as the cyclin-dependent kinase inhibitor cyclin-dependent kinase inhibitor 1 (p21) and the tumour suppressor gene phosphatase and tensin homologue (pten) thus promoting NSCs proliferation [22,24–26], and a member of the bone morphogenetic protein family 4 (bmp4) resulting in the inhibition of NSC astrocytic differentiation [27]. TLX can also bind to its own promoter thereby suppressing its transcriptional activity through a feedback loop, which can be antagonised by the SRY-box-containing Gene 2 (Sox2), which also binds to an upstream region of the TLX gene. What is more, it has been shown *in vitro* that TLX and Sox2 interact physically, whereby Sox2 acts as a transcriptional activator of TLX, to promote NSC maintenance and self-renewal [28]. Multiple small non-coding RNAs are differentially expressed in the hippocampus and a subgroup of them (miRNAs) has been shown to fine-tune the progression of adult hippocampal neurogenesis [64]. A number of miRNAs such as miR-9, miR-let7b and miR-378 have been shown to suppress TLX expression resulting in decreased NSC proliferation and accelerated neuronal differentiation [29–31]. In summary, TLX maintains NSCs in their proliferative state through a variety of autonomous and/or parallel pathways controlled by different genes, and disruption of these genes results in altered adult neurogenesis and brain plasticity [21].

Mice with spontaneous deletion of TLX present with impaired neurogenesis, synaptic plasticity, dendritic complexity and hippocampal-dependent behaviours during adulthood [32–35]. Interestingly, we have recently shown that a lack of TLX expression in the hippocampus resulted in microglial activation [35]. Our group has also previously demonstrated using both *in vitro* and *in vivo* approaches that an inverse relationship exists between levels of TLX and the microglial derived pro-inflammatory cytokine interleukin 1β (IL-1β) in the hippocampus [35,36]. Specifically, we

found a reduction in TLX expression on hippocampal NPCs *in vitro* after administration of IL-1β [36], a dramatic increase in IL-1β in the hippocampi of TLX knockout mice [35], and a protective capacity of TLX to mitigate the negative effects of IL-1β on NPCs [37]. Given that CX3CR1^{KO} mice present with increased microglial activation and hippocampal IL-1β [17] it is possible that in the absence of TLX, intracellular communication between microglia and NPCs through CX3CL1/CX3CR1 is impaired. Thus the aim of the present study was to determine whether a disturbance in CX3CL1/CX3CR1 communication mediates an impairment in TLX-related pathways (upstream regulators and downstream targets of TLX) which may have subsequent effects on hippocampal neurogenesis.

Materials and methods

Animals

Two-month-old male homozygous CX3CR1-GFP mice with CX3CR1 deficiency (CX3CR1^{KO} on C57BL/6 genetic background, n=16) and wild-type (n=16) controls were group-housed under standard housing conditions (temperature: 21°C and relative humidity: 55%), with food and water available *ad libitum*. The GFP gene was knocked-in under the CX3CR1 promoter [38]. The mice were obtained from the Jackson Laboratory (B6.129P-CX3CR1^{tm1Litt}/J; mouse strain datasheet #005582) and were generation N13F2 (backcrossed for 13 generations on C57BL/6; second filial generation was used). In order to confirm the knockout in the animals, we employed PCR and the Jackson Laboratory Protocol (Stock number: 005582) called Cx3cr1^{tm1Litt}alternate1 (see **https://www2.jax.org/protocolsdb/f?p=116:5:0::NO:5:P5_MASTER_PROTOCOL_ID,P5_JRS_CODE:27927,005582**). Two-month-old male Nr2e1$^{-/-}$ (TLX knockout) mice and wild-type controls (129S1/SvImJ background) were housed under standard housing conditions (temperature: 21°C and relative humidity: 55%), with food and water available *ad libitum*. Breeding pairs were kindly provided by Prof. Elizabeth Simpson, University of British Colombia. Nr2e1$^{-/-}$ mice exhibit a spontaneous deletion of the entire TLX allele, including all nine exons. However, the deletion of TLX does not affect the transcription of neighbouring genes [65]. All experiments were conducted in accordance with the European Directive 2010/63/EU, under an authorisation issued by the Health Products Regulatory Authority Ireland and approved by the Animal Ethics Committee of University College Cork.

BrdU administration and tissue preparation

Bromodeoxyuridine (BrdU; Sigma) was administered (4 × intraperitoneal injections over the course of 6 h at 75 μg/10 ml/kg) to the Nr2e1$^{-/-}$ and wild-type mice. Two weeks later, these mice were killed with an intraperitoneal injection of anaesthetic (0.1 ml/kg) and transcardially perfused using 0.9% phosphate buffered saline (PBS) solution followed by 4.0% paraformaldehyde (PFA) in PBS. Brains were post-fixed overnight in 4% PFA, transferred to 30% sucrose solution and subsequently flash-frozen using liquid nitrogen. Coronal sections (40 μm) through the hippocampus were collected directly on to slides in a 1:6 series, then stored at −80°C.

Immunohistochemistry

To determine the survival of adult-born hippocampal neurons in Nr2e1$^{-/-}$ mice, sections were double-labelled with BrdU and the neuronal marker NeuN. Sections were washed, DNA strands denatured by incubation in 2 M HCl for 45 min at 37°C, renatured in 0.1 M sodium tetraborate (pH 8.5) and then blocked in 3% normal donkey serum (NDS; Sigma D9663) to prevent non-specific binding. Sections were incubated with anti-BrdU antibody (Abcam; AB6326; 1:250) followed by AlexaFluor594 donkey anti-rat (Abcam; AB150156; 1:500) and NeuN (Millipore; MAB377; 1:100) antibodies. Sections were then incubated with AlexaFluor488 donkey anti-mouse antibody (Abcam; AB150105; 1:500), washed and coverslipped using anti-fade medium (DAKO; S3023). The number of microglia in the hippocampi of these animals was assessed by staining for ionised calcium binding adaptor molecule 1 (Iba-1). Sections were washed, incubated in 3% NDS and then in anti-Iba-1 antibody (Wako; 019-19741; 1:1000) overnight. Sections were then incubated in AlexaFluor488 donkey anti-rabbit antibody, counterstained with DAPI (Sigma; D9642; 1:5000) and coverslipped with anti-fade mounting medium (DAKO; S3023).

Image analysis and cell quantification

Images were obtained using an Olympus FV1000 scanning laser confocal system (Biosciences Imaging Centre, Department of Anatomy and Neuroscience, University College Cork, Ireland). Z-stack images with 1.10- or 4.4-μm step size were collected using a 10× objective (BrdU/NeuN) or 20× objective (Iba1/DAPI), respectively. The DG was imaged bilaterally on all sections. Cell quantification and area measurements were performed using the image processing software ImageJ (National Institute of Health; U.S.A.; [66]). Systematic random sampling was employed for

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all cell quantifications. For area quantification, the area of interest (i.e. the DG or the microglia soma) was outlined manually and its area calculated using ImageJ.

Total RNA extraction and cDNA synthesis

Animals were killed by cervical dislocation and the hippocampus was dissected out and stored in solution that stabilises and protects cellular RNA (RNA*later*; Sigma) for 48 h at 4°C, after which the RNAlater was removed and the tissue was frozen at −80°C until subsequent use. Samples were processed according to the GenElute kit protocol (Sigma; RTN350). Total RNA yield and purity were determined using the Nanodrop System (Thermo Scientific). Synthesis of cDNA was performed using 0.5 μg of normalised total RNA from each sample using ReadyScript cDNA synthesis mix (Sigma; RDRT-25RXN).

miRNA extraction and cDNA synthesis

Total mRNA was isolated from hippocampal samples using mirVANA miRNA Isolation Kit (Life Technologies) according to manufacturer's instructions. Total RNA yield and quality were verified using the Nanodrop2000 spectrophotometer (Thermo Scientific, Waltham, MA, U.S.A.). RNA was reverse-transcribed to cDNA using hairpin primers specific to each miRNA gene of interest on Applied Biosystem's GeneAmp PCR System 9700.

Quantitative real-time-PCR and miRNA quantification

Quantitative real-time PCR (qRT-PCR) was performed on samples in duplicate and triplicate in a 96-well plate (Applied Biosystems) and captured in real time using the StepOne Plus System (Applied Biosystems). Gene expression levels were calculated as the average CT value of three replicates for each sample relative to the expression of the housekeeper gene Tfrc, and quantified using the $2^{-\Delta\Delta CT}$ method [39]. Primer sequences were: 5′-CCCAAGTATTCTCAGATATGATTTCAA-3′ (forward) and 5′-AAAGGTATCCCTCCAACCACTC-3′ (reverse) for Tfrc; 5′-CTGGGCCCTGCAGATACTC-3′ (forward) and 5′-GGTGGCATGATGGGTAACTC-3′ (reverse) for TLX (Nr2e1); 5′-AGCCCGCTTCTGCAGGA-3′ (forward) and 5′-AAAGGCTCAGAGAAGCTGCG-3′ (reverse) for bmp4; 5′-CAGGGTTTTCTCTTGCAGAAG A-3′ (forward) and 5′-ATGTCCAATCCTGGTGATGTCCG-3′ (reverse) for p21; 5′-GTGGTCTGCCAGCTAAAGGTGA-3′ (forward) and 5′-TCAGACTTTTGTAATTTGTGAATGCT-3′ (reverse) for pten; 5′-TTAACGCAAAAACCGTGATG-3′ (forward) and 5′-GAAGCGCCTAACGTACCACT-3′ (reverse) for Sox2 and 5′-AGTGTGTCGGGTGTCCATTC-3′ (forward) and 5'- GTGCAAGCAACAGAGTTGGG -3' (reverse) for CX3CR1.

qRT-PCR was performed on the small RNA-enriched samples using probes (6 carboxy fluorescein-FAM) designed by Applied Biosystems (Carlsbad, CA, U.S.A.): miR-let7b, miR-9, miR-378. qRT-PCR was carried out on the StepOnePlus PCR machine (Applied Biosystems). Samples were heated to 95°C for 10 min, and then subjected to 40 cycles of amplification by melting at 95°C and annealing at 60°C for 1 min. Experimental samples were run in technical triplicates with 1.33 μl cDNA per reaction. To check for amplicon contamination, each run also contained template free controls for each probe used. The non-coding snRNA component U6, which is highly conserved and expressed across species, was used as the endogenous control. U6 was stably expressed in all samples and differences in miRNA expression were presented as fold change from control.

Statistical analysis

All data were analysed using SPSS statistical software (SPSS 17.0, Chicago, IL). Data were analysed by an independent-sample *t* test and an α level of 0.05 was used as criterion for statistical significance. All data are presented as mean ± S.E.M.

Results

Negative correlation between neurogenesis and microglial activation in the hippocampus as a result of TLX deficiency

There was a significant negative correlation between the mean number of BrdU/NeuN-positive cells and the mean number of Iba1-positive cells in the DG in mice with a spontaneous deletion of TLX ($Nr2e1^{-/-}$; r = 0.871, n=8, p=0.004, Figure 1A). We found the same negative correlation between cell soma size of microglia and number of BrdU/NeuN-positive cells in the absence of TLX ($Nr2e1^{-/-}$; r = 0.735, n=8, p=0.037, Figure 1B). Representative images from wild-type controls and mice with spontaneous deletion for TLX show reduced numbers of BrdU/NeuN-positive cells as well as atrophied DG morphology (Figure 1C,D), and an increased number of microglia (Figure 1E,F) in the DG of $Nr2e1^{-/-}$ mice compared with wild-type mice.

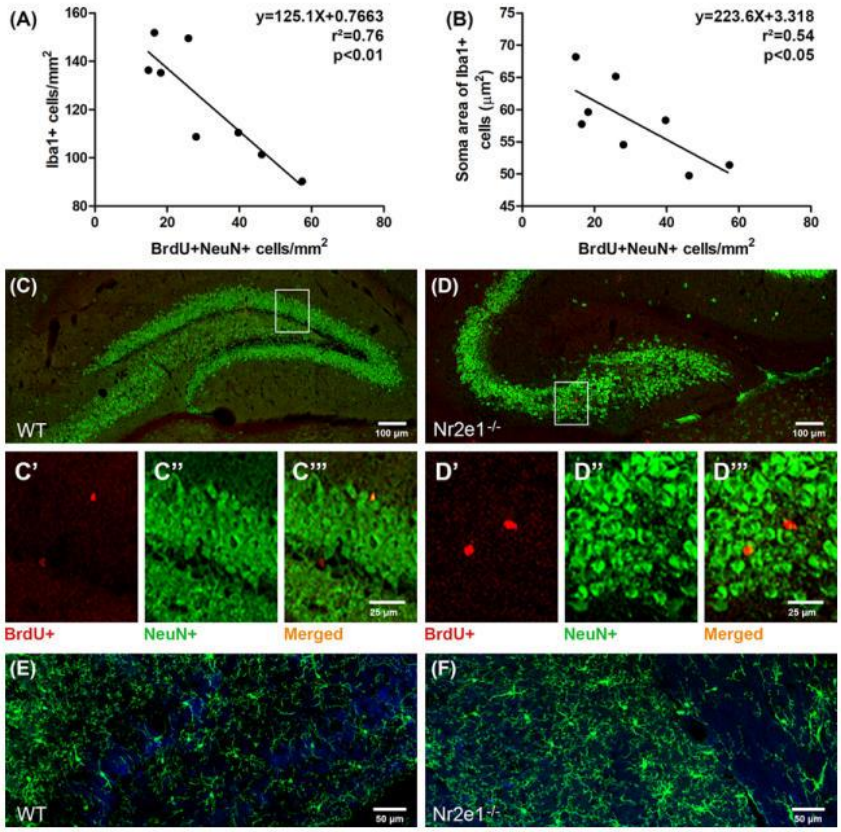

Figure 1. Negative correlation between neurogenesis and microglia activation in the hippocampi of TLX-deficient mice

Correlation between the number of Iba1+ cells and the number of BrdU+NeuN+ cells per mm^2 (**A**) and correlation between the soma area (μm^2) of Iba1+ cells and the number of BrdU+NeuN+ cells (**B**) in the DG of TLX knockout (Nr2e1$^{-/-}$) mice. Data are graphed as means (n=8). Representative images of coronal sections through the DG of wild-type (WT; (**C**)) and TLX knockout (Nr2e1$^{-/-}$; (**D**)) mice immunohistochemically stained with BrdU (red) and NeuN (green). Images were taken at 10× magnification, scale bar = 100 μm. Higher magnification images depict immunopositive cells for BrdU (**C'**,**D'**), NeuN (**C''**,**D''**) and merged channels (**C'''**,**D'''**). Scale bar = 25 μm. Representative images of coronal sections through the DG of wild-type (WT; (**E**)) and TLX knockout (Nr2e1$^{-/-}$; (**F**)) mice immunohistochemically stained with Iba1 (green) and the nuclear stain DAPI (blue). Images were taken at 20× magnification, scale bar = 50 μm.

Confirmation of the absence of CX3CR1 in the hippocampus of CX3CR1^{KO} mice

qRT-PCR analysis of the hippocampi of CX3CR1^{KO} and wild-type control mice confirmed the absence of the CX3CR1 gene in CX3CR1^{KO} mice (Figure 2).

mRNA expression of TLX but not its transcription activator Sox2 is down-regulated in the hippocampus in the absence of CX3CR1

TLX gene expression was significantly decreased in the hippocampus of CX3CR1^{KO} mice compared with controls (t (14) = 2.115, p=0.05; Figure 3A). There was no difference in expression of the TLX regulator Sox2 between wild-type

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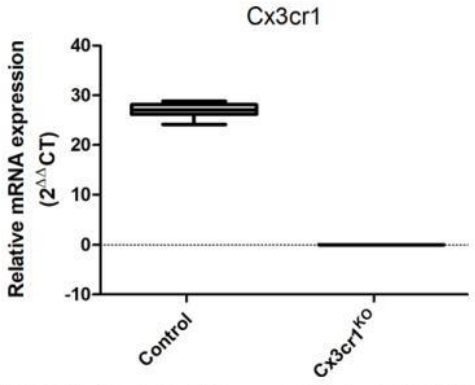

Figure 2. mRNA expression of CX3CR1 is detected in wild-type controls but not CX3CR1^{KO} mice

Relative mRNA expression of CX3CR1 in the hippocampus of wild-type controls and CX3CR1^{KO} mice. Box and whisker plots show mean, first and third quartiles, and maximum and minimum values (n=8).

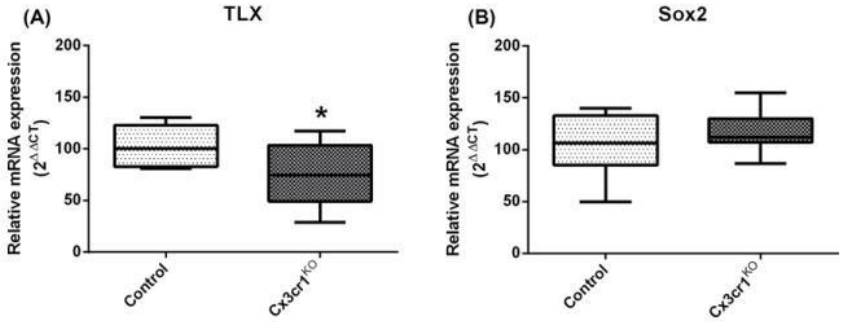

Figure 3. mRNA expression of TLX but not its transcription activator Sox2 is down-regulated in the hippocampus in the absence of CX3CR1

Relative mRNA expression of TLX (**A**) and Sox2 (**B**) in the hippocampus of wild-type control and CX3CR1^{KO} mice. All values were adjusted to the relative expression of the housekeeping gene Tfrc. Box and whisker plots show mean, first and third quartiles, and maximum and minimum values (n=8). *p≤0.05, independent-sample t test.

controls and CX3CR1^{KO} mice (Figure 3B).

Lack of CX3CR1 is not associated with changes in expression of miRNAs regulating TLX in the hippocampus

Upon examination of upstream miRNAs regulating TLX we found no change in expression of miR-let7b (Figure 4A) or miR-9 (Figure 4B) in hippocampal tissue between control and CX3CR1^{KO} mice. Nonetheless, we observed a trend towards increased expression of the TLX suppressor miR-378 in CX3CR1^{KO} mice compared with the wild-type controls (t (14) = 1.925, p=0.07; Figure 4C).

TLX target genes bmp4 and pten, but not p21 are up-regulated in the hippocampus of CX3CR1^{KO} mice

When genes targeted by TLX were measured, we detected significantly higher relative expression of bmp4 (t (14) = 2.228, p=0.04; Figure 5A) and pten (t (14) = 2.718, p=0.02; Figure 5B) in the hippocampi of CX3CR1^{KO} mice

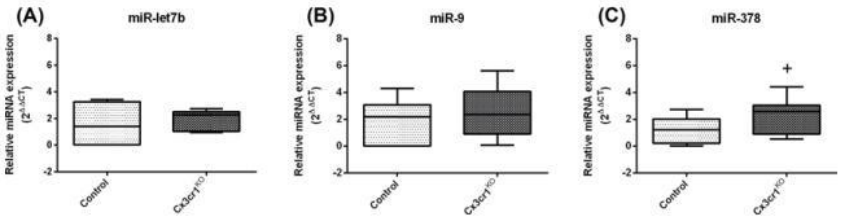

Figure 4. Lack of CX3CR1 is not associated with changes in expression of miRNAs regulating TLX in the hippocampus
miRNA expression in wild-type controls and CX3CR1^{KO} mice for miR-let7b (**A**), miR-9 (**B**) and miR-378 (**C**). Values are expressed relative to the non-coding RNA component U6. Box and whisker plots show mean, first and third quartiles, and maximum and minimum values (n=8). $^{+}$p=0.07, independent-sample t test.

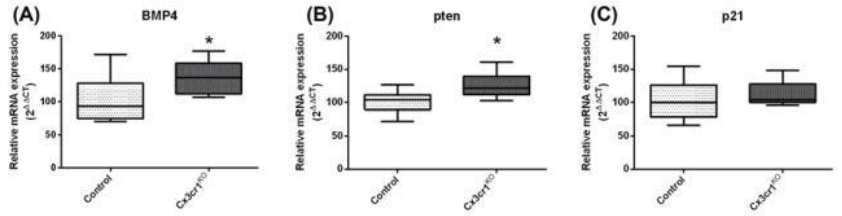

Figure 5. TLX target genes bmp4 and pten, but not p21 are up-regulated in the hippocampus of CX3CR1^{KO} mice
Relative mRNA expression of bmp4 (**A**), pten (**B**) and p21 (**C**) in the hippocampus of wild-type control and CX3CR1^{KO} mice. All values were adjusted to the relative expression of the housekeeping gene Tfrc. Box and whisker plots show mean, first and third quartiles, and maximum and minimum values (n=8). *p<0.05, independent-sample t test. Abbreviation: bmp4, bone morphogenic protein 4.

compared with wild-type controls. There was no alteration in relative expression of the cyclin-dependent kinase inhibitor gene p21 another target of TLX, between the hippocampi of wild-type controls and the CX3CR1^{KO} mice (Figure 5C).

Discussion

Here we have shown that in the absence of CX3CR1, TLX transcription within the hippocampus is reduced. This reduction in TLX relative mRNA abundance is not associated with changes in miR-let7b, miR-9 or miR-378, the upstream repressors of the gene, though there was a trend towards increased expression of the TLX repressor miR-378. Furthermore, no change was observed in the expression of the TLX promoter and activator Sox2. However, CX3CR1^{KO} mice exhibited an increase in bmp4 expression, a downstream target of TLX, which is involved in gliogenesis and astrocyte differentiation. In addition, in the absence of CX3CR1, hippocampal expression of the TLX repressor target pten but not p21 was increased. Collectively, these data suggest that absence of CX3CR1 promotes down-regulation of TLX expression and its downstream targets, without influencing the regulators of the gene. Thus TLX may be an important target in the cross-talk between microglia and hippocampal NSCs.

We hypothesise that the CX3CR1 signalling pathway in microglia, and TLX signalling pathways in NPCs interact to maintain homeostasis in the neurogenic niche in the adult hippocampus, which is supported by our observation of a decrease in TLX transcription in CX3CR1^{KO} mice. This is also in-line with the emerging evidence that microglia regulate neurogenesis in a temporal- and spatial-dependent manner, and that microglia are proposed as a key mediator and integrator of information that may influence the neurogenic niches (reviewed in [4]). Here we show a significant negative correlation between hippocampal neurogenesis and microglia number and soma size in the absence of TLX, which supports our previous demonstration that the absence of TLX results in an activated microglial phenotype, increased levels of endogenous IL-1β and impaired hippocampal neurogenesis [35], and thus implicate TLX as a mediator of NS/PC-microglial communication. However, the temporal relationship between the associations we observed remains unclear. Further studies examining if NPCs in the hippocampi of TLX-deficient mice

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have impaired CX3CL1 would provide valuable information on whether the activated microglia in TLX knockout mice has been promoted through a CX3CL1/CX3CR1-mediated pathway. Isolating microglia from the hippocampi of TLX-deficient mice and examining how these microglia behave in response to pro- and anti-inflammatory stimulation with cytokines for various durations would provide insight into the temporal relationship of our findings. Moreover, since CX3CR1-deficient mice have been shown to present with impaired hippocampal-dependent but not olfactory-dependent memory performance [17,19], it would be of interest to compare the expression of TLX and other neurogenic markers in these two neurogenic niches (the SGZ and the subventricular zone) in CX3CR1^{KO} mice. This would thus illustrate whether TLX mediates NPC–microglia cross-talk in a region-specific manner.

While it has been demonstrated that microglia have a direct effect on NSCs, the mechanisms by which microglia exercise their influence on NSCs remain largely unknown [2,4,58]. Our findings suggest that deletion of CX3CR1 expression on microglia facilitate the activation of down- but not up-stream pathways of TLX. As there was no up-regulation in mRNA expression of the transcription factor Sox2 in the hippocampi of CX3CR1^{KO} mice, it is possible that the reduction in TLX gene transcription that we observed is regulated through a self-repression mechanism. This is supported by the fact that the transcriptional regulation of NPCs by Sox2 and TLX is autonomous such that both can act independently for the potentiation of cell proliferation and the repression of cell differentiation in order to maintain the undifferentiated and self-renewable state of progenitors within the neurogenic niche [40,41].

A number of miRNAs, such as miR-9, miR-137, miR-let7d and miR-let7b, have been shown to regulate neurogenesis rate and progression through suppression of TLX *in vitro* [29,31,42–45] and are associated with suppression of TLX expression *in vivo* in hippocampi of adult mice [46]. Thus we examined whether the reduction in TLX gene transcription in adult CX3CR1^{KO} mice was associated with up-regulation of miR-let7b and/or miR-9. Our results showed no change in the expression of either miR-let7b or miR-9 in the absence of CX3CR1 in the hippocampi of knockout and wild-type mice. It has been shown that miR-let7b and miR-9 are heterochronic switch genes, which induce acceleration in NSC differentiation and reduction in their proliferation by targeting TLX expression [29,30]. Interestingly, both miRNAs not only bind to TLX, but their expression is inversely related to that of TLX such that silencing and overexpression of the miRNAs causes an inverse increase or reduction, respectively, in TLX [29,30]. This phenomenon has been demonstrated in adult hippocampal NPC cultures and in the embryonic developing brain for both miR-9 and miR-let7b [29,30] as well as in retinal progenitor cells for miR-let7b [45]. As these processes may occur autonomously in increased NPC neuronal differentiation (which would be induced by miR-let7b and/or miR-9) combined with the fact we did not observe any change in miR-let7b and/or miR-9 expression, it follows that the reduction in TLX in CX3CR1^{KO} mice is independent of either the TLX-miR-let7b regulatory loop or the TLX-miR-9 feedback pathway. This further supports our conjecture that the down-regulation of TLX observed in the absence of CX3CR1 could be a result of a self-repression mechanism, thereby positioning TLX as a potential target or co-regulator of the CX3CR1/CX3CL1 pathway.

miR-378 has been shown as an important enhancer of cell survival through reduction in apoptosis [47,48], reduction in cell proliferation and promotion of differentiation [31]. We observed a trend towards increased expression of miR-378 in the hippocampus of CX3CR1^{KO} mice compared with controls. Similar to miR-let7b and miR-9, miR-378 has been shown to exert its effect on NSCs through binding and suppressing the expression of TLX [31] coupled with an up-regulation of two TLX downstream targets – p21 and pten [31]. Given the trend towards increased miR-378 expression, we examined the mRNA levels of p21 and pten and observed an increase in pten but no change in p21 expression in the hippocampi of CX3CR1^{KO} mice. However, a limitation of the present study is that we examined expression levels in the whole hippocampus, rather than specifically in the DG where NSCs predominantly reside. The mechanisms by which TLX suppresses both of these genes have been extensively studied: the repression of both genes occurs through an interaction between TLX and the histone demethylase lysine-specific histone demethylase 1A (LSD1) [49,50]. In the case of pten, however, the histone deacetylases (HDAC) 1 (HDAC1) and 2 (HDAC2) are recruited to form a complex with the REST corepressor 1 (CoREST), which results in demethylation of trimethylation of histone H3 at lysine 4 (H3K4) at the proximal region of pten and hence its suppression [51] (Figure 6). p21 repression on the other hand, can result from the interaction between TLX and LSD1 with HDAC5 [22] or from tumour protein TP53 (p53)-TLX-dependent signalling [24] (Figure 6). Within NSCs, it has been shown that blocking TLX-mediated suppression of both pten and p21 resulted in a reduction in NSC proliferation [22] and an increase in quiescent hippocampal NSCs rather than an increase in differentiating NSCs, which was coupled with activation of pten and p21 signalling pathways [24]. Thus in the context of the present study, in the absence of CX3CR1 on microglia TLX expression in NPCs is reduced, which is associated with a trend towards increased expression of the TLX suppressor miR-378 and an increase in the TLX downstream target pten but not p21. Hence, it is possible that the reduction in hippocampal neurogenesis observed in CX3CR1^{KO} mice by others [17,19] results from activation of TLX-suppressing signalling pathways that inhibit activation of quiescent NSCs and maintain them in their

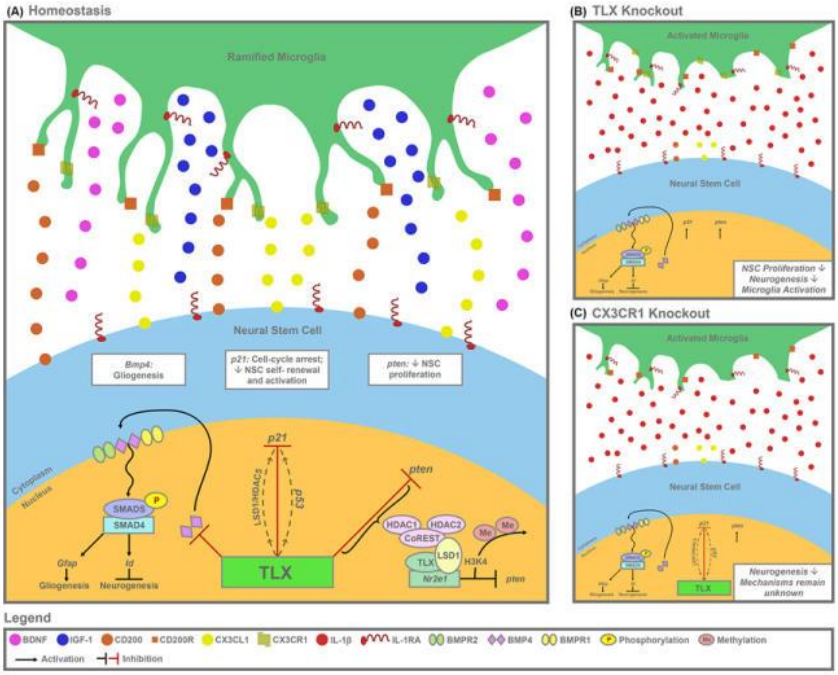

Figure 6. Schematic diagram illustrating possible mechanisms by which NSCs and microglia interact

Under homoeostasis (**A**), TLX acts as a transcriptional repressor of a variety of genes in order to maintain NSCs in their proliferative and non-differentiative states by inhibiting gliogenesis (bmp4 pathway), cell-cycle arrest (p21 pathway) and decreased proliferation (pten pathway). Microglia send neurotrophic signals such as BDNF and IGF-1 to NSCs, and receive output from NSCs through CX3CL1/CX3CR1- and CD200/CD200R-mediated pathways. In the TLX knockout mouse (**B**), microglia become activated (retracted processes coupled with increased levels of endogenous IL-1β) and NSC proliferation and neurogenesis decreases. In the CX3CR1 knockout mouse (**C**), microglia activation and increased levels of endogenous IL-1β are coupled with reduced TLX expression, up-regulated levels of bmp4 and pten and a reduction in neurogenesis. GFAP: Glial fibrillary acidic protein; Id: Inhibitor of DNA-binding/differentiation protein; BMPR1: Bone morphogenic protein receptor, type 1; bmp4, Bone morphogenic protein 4; BMPR2: Bone morphogenic protein receptor, type 2; SMADS: homologies to the *Caenorhabditis elegans* SMA ('small' worm phenotype) and *Drosophila* MAD ('Mothers Against Decapentaplegic') family of genes; P: phosphorylation; SMAD4: Mothers against decapentaplegic homologue 4; CoREST: REST (RE1-silencing transcription factor) corepressor 1; TLX: Protein Tailless Homologue; Nr2e1: Nuclear receptor subfamily 2 Group E Member 1; Me: methylation.

non-proliferative state through pten signalling mechanisms. Interestingly, it has been shown that attenuation of pten increases p21 stability in cancer stem cells [55], which may explain why we observed an increase in pten only but not in p21.

TLX is also involved in the transcriptional repression of the bone morphogenic protein 4 (bmp4)-SMAD signalling pathway which activates astrogenesis [27] (Figure 6). Specifically, TLX prevents the binding of bmp ligands to their type I (BMPR1; Figure 6) and type II (BMPR2; Figure 6) receptors; blocking the activation of these receptors prevents the phosphorylation of regulatory SMADS (Mothers against decapentaplegic homologue 4) and their dimerisation with the common cofactor SMAD4 (Figure 6; [27]). Under normal physiological conditions, the SMAD complex transcriptionally activates downstream targets which promote astrogenesis (glial fibrillary acidic protein (GFAP)) and inhibits neurogenesis via suppressing inhibitors of differentiation (inhibitor of DNA-binding/differentiation proteins (Ids); Figure 6; [27]). Interestingly, in the present study CX3CR1^{KO} mice displayed an increase in relative mRNA

expression of bmp4, the downstream target of TLX, responsible for astrogenesis. Thus it would be of interest to examine in future studies whether the reduction in hippocampal neurogenesis in CX3CR1^{KO} mice is coupled with an increase in hippocampal astrogenesis. This is of particular importance, given that we previously observed an increased level of endogenous IL-1β coupled with increased microglia activation in TLX knockout mice [35]. Similarly, CX3CR1-deficient mice present with increased microglia activation and increased endogenous hippocampal IL-1β [17,19]. Due to the fact that astrocytes are major producers of IL-1β in the central nervous system [56,57], they may act as the 'middle man' in the cascade leading to impaired neurogenesis as a result of CX3CR1 and/or TLX deficiency. Thus, studies investigating the cause of inflammation in the absence of TLX will be key to determining the relationship between TLX and CX3CL1/CX3CR1 signalling.

It has previously been shown that microglia development and adult hippocampal neurogenesis are impaired in CX3CR1^{KO} mice [17,52], but has more recently been suggested that the decrease in neurogenesis observed in the CX3CR1-deficient mice is due to pathways independent of CX3CL1 [53]. Thus understanding the precise interactions and signalling mechanisms between and within microglia and NPCs may aid our understanding of diseases associated with defective microglia–neuronal cross-talk as well as with the neuropathology of aging [54]. Here we show that CX3CR1 deficiency in the hippocampus leads to the activation of TLX-dependent pathways within NSCs that may inhibit their self-renewal and promote their adoption of an astrocytic fate. We propose that TLX is a mediator in maintaining homoeostasis between microglia and NPCs. However, future studies are needed to examine whether TLX repression leads to impaired proliferation and neurogenesis and/or gliogenesis through CX3CL1/CX3CR1-dependent mechanisms.

Competing interests

The authors declare that there are no competing interests associated with the manuscript.

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Author contribution

D.A.K., Y.M.N. and J.F.C. conceived and designed the experiments. D.A.K., G.M.M., A.E.H. and V.R. performed the experiments and analysed the data. D.A.K. and Y.M.N. co-wrote the paper. K.N., J.F.C. and Y.M.N. supervised the work.

Abbreviations

BMP4, bone morphogenic protein 4; BrdU, bromodeoxyuridine; CX3CL1, fractalkine/chemokine (C-X3-C motif) ligand 1; CX3CR1, chemokine receptor 1; DCX, doublecortin; DG, dentate gyrus; HDAC, histone deacetylase; Iba-1, ionised calcium binding adaptor molecule 1; IL-1β, interleukin 1β; LSD1, lysine-specific histone demethylase 1A; NDS, normal donkey serum; NPC, neural precursor cell; Nr2e1, nuclear receptor subfamily 2 group E member 1; NSC, neural stem cell; p21, cyclin-dependent kinase inhibitor 1; PFA, paraformaldehyde; pten, phosphatase and tensin homologue; SGZ, subgranular zone; Sox2, sex-determining region Y- box2; TLX, protein tailless homologue.

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RAPID COMMUNICATION

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Deletion of TLX and social isolation impairs exercise-induced neurogenesis in the adolescent hippocampus

Danka A. Kozareva[1,2] | Olivia F. O'Leary[1,2] | John F. Cryan[1,2] | Yvonne M. Nolan[1,2]

[1]Department of Anatomy and Neuroscience, University College Cork, Ireland
[2]APC Microbiome Institute, University College Cork, Ireland

Correspondence
Yvonne M. Nolan, Department of Anatomy and Neuroscience, University College Cork, Ireland.
Email: y.nolan@ucc.ie

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Abstract

Adolescence is a sensitive period of neurodevelopment during which life experiences can have profound effects on the brain. Hippocampal neurogenesis, the neurodevelopmental process of generating functional new neurons from neural stem cells, occurs throughout the lifespan and has been shown to play a role in learning, memory and in mood regulation. In adulthood it is influenced by extrinsic environmental factors such as exercise and stress. Intrinsic factors that regulate hippocampal neurogenesis include the orphan nuclear receptor TLX (Nr2e1) which is primarily expressed in the neurogenic niches of the brain. While mechanisms regulating adult hippocampal neurogenesis have been widely studied, less is known on how hippocampal neurogenesis is affected during adolescence. The aim of this study was to investigate the influence of both TLX and isolation stress on exercise-induced increases in neurogenesis in running and sedentary conditions during adolescence. Single- (isolation stress) wild type and Nr2e1$^{-/-}$ mice or pair-housed wild type mice were housed in sedentary conditions or allowed free access to running wheels for 3 weeks during adolescence. A reduction of neuronal survival was evident in mice lacking TLX, and exercise did not increase hippocampal neurogenesis in these Nr2e1$^{-/-}$ mice. This suggests that TLX is necessary for the pro-neurogenic effects of exercise during adolescence. Interestingly, although social isolation during adolescence did not affect hippocampal neurogenesis, it prevented an exercise-induced increase in neurogenesis in the ventral hippocampus. Together these data demonstrate the importance of intrinsic and extrinsic factors in promoting an exercise-induced increase in neurogenesis at this key point in life.

KEYWORDS
adolescence, adult neurogenesis, exercise, stress, TLX

1 | INTRODUCTION

Adolescence is a critical phase of development associated with plasticity-driven organization of neural circuits in the hippocampus, prefrontal cortex, and amygdala (Pattwell, Bath, Casey, Ninan, & Lee, 2011; Selemon, 2013). It is also a key period for susceptibility to stress and the emergence of neurobiological disorders such as schizophrenia, depression, and anxiety (Green & Nolan, 2014; Hueston, Cryan, & Nolan, 2017; O'Connor & Cryan, 2014; Paus, Keshavan, & Giedd, 2008). By approximately postnatal day (P) 30 in rodents, dentate gyrus (DG) formation, cerebellar neurogenesis, and myelogenesis are completed, and neurogenesis (the birth of new neurons) is restricted to the niches of the brain where the process persists through adulthood—the subgranular zone (SGZ) of the DG of the hippocampus and the subventricular zone (Lemasson, Saghatelyan, Olivo-Marin, & Lledo, 2005; Li, Mu, & Gage, 2009).

Hippocampal neurogenesis has been widely studied in the adult brain and is known to be regulated by several extrinsic and intrinsic factors (Aimone et al., 2014; Gregoire, Bonenfant, Le Nguyen, Aumont, & Fernandes, 2014). For example, extrinsic factors such as stress and exercise have been shown to decrease or increase adult hippocampal neurogenesis, respectively (Levone, Cryan, & O'leary, 2015; van Praag, Christie, Sejnowski, & Gage, 1999). However, comparatively fewer studies have investigated the impact of these extrinsic factors on hippocampal neurogenesis in the adolescent brain (Abel & Rissman, 2013; Kirshenbaum, Lieberman, Briner, Leonardo, & Dranovsky, 2014; Wei, Meaney, Duman, & Kaffman, 2011). In adult rodents, exercise has been shown to enhance learning and memory (Creer, Romberg, Saksida, van

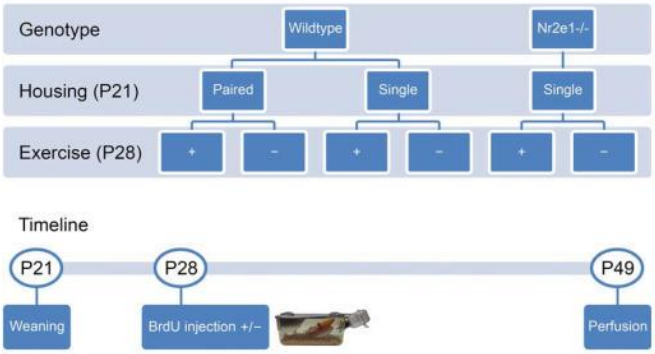

FIGURE 1 Experimental design. Outline of the experimental groups and timeline illustrating the duration of the experiment [Color figure can be viewed at wileyonlinelibrary.com]

Praag, & Bussey, 2010; Marlatt, Potter, Lucassen, & van Praag, 2012; van Praag, Christie, et al., 1999), protect against stress-induced depression and anxiety-like behaviors (Duman, Schlesinger, Russell, & Duman, 2008; Grippo et al., 2014) and protect against cognitive deficits in neurodegenerative disorders (Barbour, Edenfield, & Blumenthal, 2007; Cotman, Berchtold, & Christie, 2007; Ryan & Nolan, 2016). How exercise can facilitate processes as diverse as spatial learning and memory, anxiety and responses to stress is not yet clear. However, accumulating evidence suggests that the hippocampus is functionally segregated along its dorsoventral axis in rodents such that the dorsal hippocampus (dHi) plays a predominant role in spatial learning and memory while the ventral hippocampus (vHi) plays a predominant role in anxiety and the stress response (Bannerman et al., 2004; Fanselow & Dong, 2010). Similarly, there is an emerging view that neurogenesis might also be similarly functionally segregated along this axis (O'Leary & Cryan, 2014; Tanti & Belzung, 2013). Intrinsic factors that control hippocampal neurogenesis include the orphan nuclear receptor TLX (Nr2e1) which is primarily expressed in the neurogenic niches of the postnatal brain and has been implicated as an important regulator of neural stem cells by maintaining them in their proliferative and nondifferentiated state (Roy et al., 2004; Shi et al., 2004). In a spontaneous deletion mouse model, adult TLX knock out ($Nr2e1^{-/-}$) mice display altered neurogenesis and synaptic plasticity, as well as an impairment of dendritic structures in the DG. These mice also present with an aggressive phenotype and display cognitive impairments in hippocampal-dependent tasks (Christie et al., 2006; Young et al., 2002). Interestingly, some of these effects are apparent in adolescence (O'Leary, Kozareva, et al., 2016; O'Leary, O'Leary, Cryan, & Nolan, 2016).

Taken together, while mechanisms regulating adult hippocampal neurogenesis have been thoroughly investigated, less is known about how neurogenesis is affected during the adolescent period. Moreover, the impact of facilitators and impeders of neurogenesis, such as exercise and stress, on the adolescent brain has yet to be established. This is surprising given that adolescence is a critical period for the maturation of neurons as well as a time during which profound social and physiological change occurs. Thus, the aim of this study was to investigate (a) the impact of exercise during adolescence on hippocampal neurogenesis; (b) the role of the intrinsic factor TLX on exercise-induced changes in hippocampal neurogenesis and (c) whether social isolation stress influences exercise-induced changes in hippocampal neurogenesis. Given the growing evidence of segregated effects on neurogenesis along the dorsoventral axis of the hippocampus, we examined the impact of exercise, TLX, and social isolation on neurogenesis in the dorsal versus ventral hippocampus.

This animal study was conducted in strict compliance with the European Directive 2010/63/EU, and under an authorization issued by the Health Products Regulatory Authority Ireland and approved by the Animal Ethics Committee of University College Cork (UCC). Breeding pairs of $Nr2e1^{-/-}$ mice exhibiting a spontaneous deletion of TLX were kindly provided by Prof. Elizabeth Simpson (University of British Colombia) and were generated as previously described (Wong et al., 2010). Male $Nr2e1^{-/-}$ mice and wild type (WT) littermate controls on a BL6129 background were single-housed on weaning and given *ad libitum* access to food and water under a 12-12 hr light/dark cycle. At 4 weeks of age (P28), mice received 4 × bromodeoxyuridine (BrdU; 75mg/kg, i.p., Sigma Cat# B5002) injections at 2-hour intervals to label newly-born cells. Half of the animals from each genotype were then housed with free access to a running wheel for 3 weeks (Med Associates Inc Cat# ENV-044), thus there were 4 experimental groups (WT sedentary, WT running, $Nr2e1^{-/-}$ sedentary; $Nr2e1^{-/-}$ running; $n = 5–6$ per group; Figure 1). Male mice were collected from 11 litters in total. For welfare reasons, $Nr2e1^{-/-}$ mice have to be single housed due to their aggressive phenotype (Young et al., 2002). Thus, their corresponding WT littermates were also single housed. As single housing is a social isolation stressor, we sought to examine whether this chronic stress influenced the effect of exercise on hippocampal neurogenesis in WT mice (Figure 1).

Three weeks following BrdU administration and initiation of exercise, mice (P49) were anesthetized with Euthasol (1.0 mL/kg, i.p.) and transcardially perfused with phosphate buffered saline (PBS) solution followed by 4.0% paraformaldehyde (PFA). Brains were postfixed overnight, cryoprotected in 30% sucrose and subsequently flash frozen.

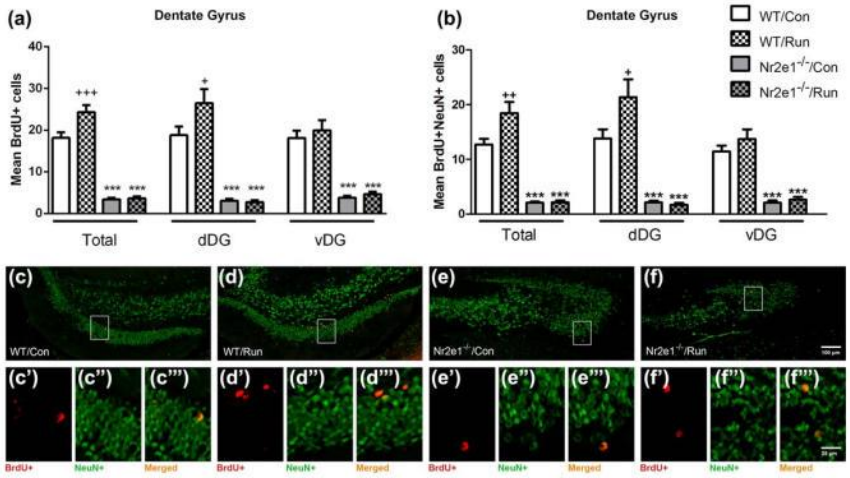

FIGURE 2 Nr2e1 is necessary for the pro-neurogenic effect of exercise. Mean number of BrdU+ (a) and BrdU+NeuN+ (b) cells per section in the whole, dorsal and ventral hippocampus of single-housed wild type or Nr2e1$^{-/-}$ adolescent mice with or without access to running wheels. Data are expressed as mean ± *SEM*. *** $p < .001$ compared to WT counterparts; ++ $p < .01$, + $p < .05$ compared to WT sedentary mice; (Two-way ANOVA, Fisher's LSD), $n = 5$–6. Representative confocal images through the DG from WT sedentary (c) and running (d), and Nr2e1$^{-/-}$ sedentary (e) and running (f) mice. Immunohistochemical staining shows BrdU+ (red), NeuN+ (green) and BrdU+NeuN+ (orange) cells at 10X magnification. Scale bar = 100 μm. Higher magnification images depict immunopositive cells in the DG for BrdU (c' – f'), NeuN (c'' – f'') and merged channels (c''' – f'''). Scale bar = 25 μm [Color figure can be viewed at wileyonlinelibrary.com]

Coronal sections (40 μm) through the entire hippocampus were collected onto slides in a 1:6 series and surviving cells and surviving newly born neurons were identified using immunohistochemistry for BrdU alone, and colocalization of BrdU and the neuronal marker NeuN, respectively. Briefly, sections were incubated in HCl (2M; 37°C, 45 mins), renatured in 0.1 M sodium tetraborate (pH 8.5), and then blocked in 3% normal donkey serum (NDS; Sigma Cat# D9663). Sections were incubated with rat anti-BrdU antibody (Abcam Cat# AB6326; 1:250; overnight at 4°C), followed by AlexaFluor594 donkey anti-rat (Abcam Cat# ab150156; 1:500; 2 hrs at room temperature) and mouse anti-NeuN (Millipore Cat# MAB377; 1:100; overnight at 4°C). Sections were then incubated in AlexaFluor488 donkey anti-mouse (Abcam Cat# ab150105 1:500; 2 hrs at room temperature). To assess cell death, immunohistochemistry for the apoptosis marker caspase-3 was performed. Briefly, sections were incubated in H_2O_2 (in 1% methanol, 40 mins at room temperature) and then blocked in 10% normal goat serum (NGS; Sigma Cat# G9023). Sections were incubated with rabbit anti-active caspase-3 antibody (Promega Cat# G7481; 1:250; overnight at 4°C), followed by sequential incubations with the streptavidin-biotin immunoenzymatic antigen detection system (Abcam, Cat# ab64261). Images were obtained using an Olympus BX533 upright microscope coupled to an Olympus DP72 camera and Olympus FV1000 scanning laser confocal system (BioSciences Imaging Centre, UCC). Immunofluorescent Z-stack images with a 4.4 μm step size were collected using a 10× objective, while DAB staining was analyzed at 20× magnification with bright field. Systematic random sampling was used through the whole DG by counting the cells on both hemispheres of each section in 1:6 series (240 μm apart). Cell quantification was performed using the image processing software package, ImageJ v 1.43m. All cell numbers are expressed as an average per section. The dorsal DG was defined as AP: −0.94 to AP: −2.30 and the ventral DG as AP: −2.46 to AP: −3.80 as described previously (O'Leary, O'connor, & Cryan, 2012; Paizanis et al., 2010).

Exercise significantly increased the number of surviving new cells in the whole DG of WT mice (exercise effect: $F_{1,19} = 7.915$, $p = .011$), but not in Nr2e1$^{-/-}$ mice (exercise × genotype interaction: $F_{1,19} = 6.747$, $p = .018$; Figure 2a). Furthermore, both sedentary and running Nr2e1$^{-/-}$ mice exhibited a reduction in the number of surviving new cells compared to their WT littermates (genotype effect: $F_{1,19} = 244.002$, $p < .001$; Figure 2a). These changes in cell survival were driven by an exercise-induced increase in the number of surviving new neurons (neurogenesis) in the DG of WT mice (exercise effect: $F_{1,19} = 6.024$, $p = .024$), but not in Nr2e1$^{-/-}$ mice (exercise × genotype interaction: $F_{1,19} = 5.704$, $p = .027$; Figure 2b). Additionally, sedentary Nr2e1$^{-/-}$ mice also exhibited reduced hippocampal neurogenesis compared to their WT littermates (genotype effect: $F_{1,19} = 130.096$, $p < .001$; Figure 2b). On subdivision of the DG into dorsal (dDG) and ventral (vDG) regions, this genotype effect was apparent in both subregions for both new cell (BrdU+) and new neuron (BrdU + NeuN+) survival (**BrdU+**: dDG: $F_{1,19} = 98.723$, $p < .001$; vDG: $F_{1,19} = 92.748$, $p < .001$; Figure 2a; **BrdU + NeuN+**: dDG: $F_{1,19} = 69.635$, $p < .001$; vDG: $F_{1,19} = 88.056$, $p < .001$; Figure 2b). However, the exercise-

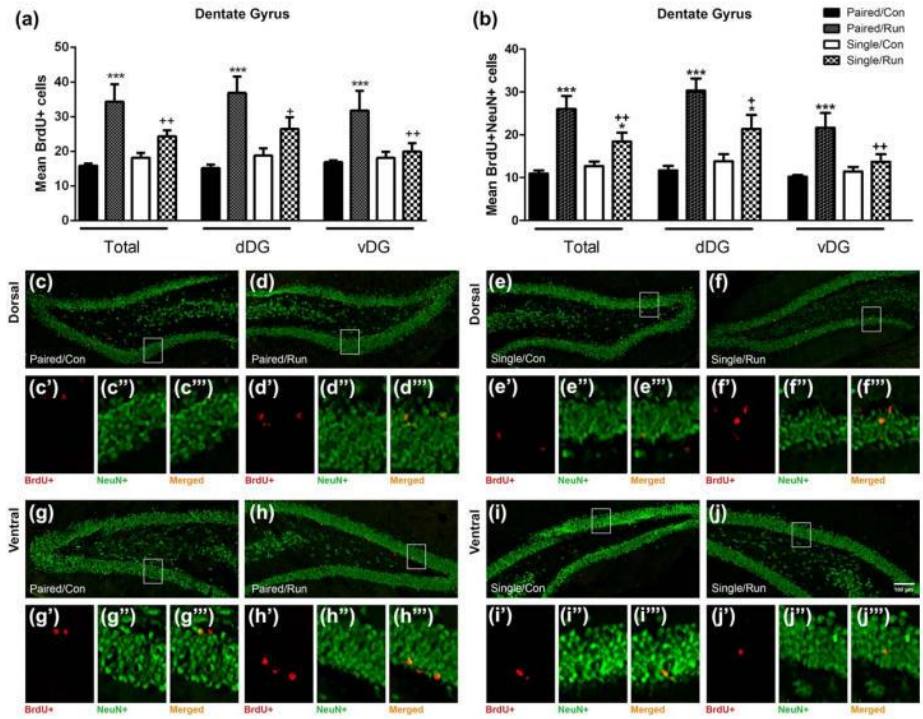

FIGURE 3 Differential modulation of neurogenesis by isolation stress and exercise across the septotemporal axis of the DG in adolescent mice. Mean number of BrdU+ (a) and BrdU+NeuN+ (b) cells per section in the whole, dorsal and ventral hippocampus of single- or pair-housed adolescent mice with or without access to running wheels. Data are expressed as mean ± *SEM*. $^{*}p < .05$, $^{***}p < .001$ compared to single- or pair-housed sedentary counterparts; $+p < .05$, $++\ p < .01$ compared to pair-housed running mice; (Two-way ANOVA, Fisher's LSD), $n = 4–6$. Representative confocal images of coronal sections through the dDG and vDG immunohistochemically stained with BrdU (red) and NeuN (green) from pair-housed sedentary (dDG: c, vDG: g), pair-housed running (dDG: d, vDG: h), single-housed sedentary (dDG: e, vDG: i) and single-housed running (dDG: f, vDG: j) mice. Images were taken at 10X magnification. Scale bar = 100 μm. Higher magnification images depict immunopositive cells in the DG for BrdU (dDG: c' – f', vDG: g' – j'), NeuN (dDG: c'' – f'', vDG: g'' – j'') and merged channels (dDG: c''' – f''', vDG: g''' – j'''). Scale bar = 25 μm [Color figure can be viewed at wileyonlinelibrary.com]

induced increase in new cell survival and neurogenesis was only apparent in the dDG (exercise × genotype interaction: **BrdU+**: $F_{1,19} = 4.078$, $p = .058$; **BrdU + NeuN+**: $F_{1,19} = 4.502$; $p = .047$) but not in the vDG of WT mice (Figure 2). No difference was observed in the percentage of surviving new cells that adopted a neuronal fate in the DG of WT animals; almost all BrdU+ cells were immunopositive for NeuN and there was no effect of exercise on cells adopting a neuronal fate. In the DG of Nr2e1-/- mice, however, a significantly smaller percentage of surviving cells matured into neurons in both sedentary and exercise conditions (genotype effect: $F_{1,19} = 31.530$, $p < .001$; % BrdU + NeuN+ cells/BrdU+ cells: WT sedentary: $M = 70.156$, $SD = 4.065$; WT running: $M = 74.398$, $SD = 9.338$; Nr2e1-/- sedentary: $M = 53.839$, $SD = 9.130$; Nr2e1-/- running: $M = 51.151$, $SD = 9.257$).

As exercise increased neurogenesis in the dorsal but not ventral DG of WT mice, we decided to investigate whether the stress of this social isolation during adolescence could explain the lack of effect of exercise-induced increases in neurogenesis in the vDG. Thus, we compared the effect of running on cell survival and hippocampal neurogenesis in single-housed compared to pair-housed WT mice (Figure 3). Two-way ANOVA revealed that exercise significantly increased cell survival in paired-housed but not single-housed mice in the whole DG, and this effect persisted in both dDG and vDG (exercise × stress interaction: total DG: $F_{1,17} = 7.420$, $p = .014$; dDG: $F_{1,17} = 5.911$, $p = .026$; vDG: $F_{1,17} = 5.825$, $p = .027$; Figure 3a). Interestingly, exercise significantly increased neurogenesis of both paired and single-housed mice who had access to a running wheel for 3 weeks compared to their sedentary counterparts (exercise effect: DG: $F_{1,17} = 34.054$; $p < .001$). However, the exercise-induced increase in neurogenesis was significantly lower in single-housed animals than in pair-housed animals (exercise × stress interaction: DG: $F_{1,17} = 6.866$; $p = .018$). Moreover, on analysis of the dorsal and ventral regions of the DG, we found that although exercise increased neurogenesis in the dDG in both pair-

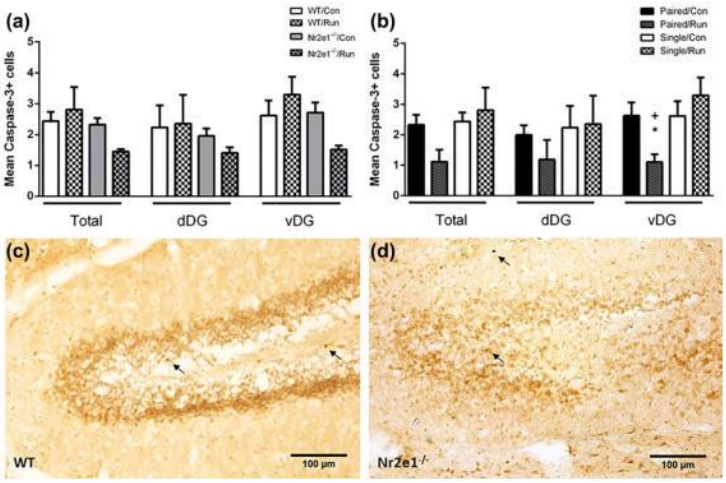

FIGURE 4 Differential modulation of apoptosis by isolation stress and exercise across the septotemporal axis of the DG in WT but not Nr2e1$^{-/-}$ adolescent mice. Mean number of active caspase-3+ cells per section in whole, dorsal and ventral hippocampus of singly-housed wild type or Nr2e1$^{-/-}$ adolescent mice (a) and single- or pair-housed wild type adolescent mice with or without access to running wheels (b). Data are expressed as mean ± *SEM*. *$p < .05$, compared to single- or pair-housed sedentary counterparts; +$p < .05$, compared to single-housed running mice; (Two-way ANOVA, Fisher's LSD), $n = 4$. Representative bright field images of coronal sections through the hippocampus immunocytochemically stained with active caspase-3 (dark brown; black arrows) from a wild type control (c) and Nr2e1$^{-/-}$ (d) mouse. Images were taken at 20X magnification. Scale bar = 100 µm [Color figure can be viewed at wileyonlinelibrary.com]

housed and single-housed mice (dDG: $F_{1,17} = 29.350$; $p < .001$; Figure 3b), this effect was attenuated by the stress of single housing (exercise × stress interaction dDG: $F_{1,17} = 5.239$, $p = .035$). Additionally, the exercise-induced increases in neurogenesis observed in the vDG of pair-housed mice (exercise effect: vDG: $F_{1,17} = 15.421$; $p = .001$) was prevented in single housed mice (exercise × stress interaction: vDG: $F_{1,17} = 6.933$, $p = .017$; Figure 3b).

There was a trend toward a reduction of caspase-3-positive cells in the vDG of Nr2e1$^{-/-}$ mice with access to a running wheel, compared to their sedentary and WT running counterparts (exercise × genotype interaction: $F_{1,12} = 3.8$, $p = .077$; Figure 4a). There was a significant reduction in the number of apoptotic cells in the vDG but not dDG or whole DG in pair-housed mice with access to a running wheel compared to their single-housed or pair-housed sedentary littermates (exercise × stress interaction: vDG: $F_{1,12} = 5.770$, $p < .05$; Figure 4b).

Our results indicate that the well-known pro-neurogenic effect of exercise observed during adulthood (van Praag, Christie, et al., 1999; van Praag, Kempermann, & Gage, 1999) also occurs in adolescent male mice. This finding corroborates the effects of exercise on cognitive function observed in adolescent rats (Hopkins, Nitecki, & Bucci, 2011). Mice exposed to exercise during adolescence have increased hippocampal levels of pro-neurogenic brain-derived neurotrophic factor (BDNF) (Gallego, Cox, Funk, Foster, & Ehringer, 2015) and enhanced expression of synaptic plasticity genes (Abel & Rissman, 2013). Exercise has also been shown to rescue alcohol-induced deficits in cell proliferation in adolescent rats (Helfer, Goodlett, Greenough, & Klintsova, 2009). Interestingly, a physical skills training task has recently been reported to increase cell survival in the DG of adolescent rats (DiFeo & Shors, 2017). However, training on this type of tasks involves physical exercise and learning, both of which increase neurogenesis and so a definitive conclusion on the effects of exercise alone during adolescence on hippocampal neurogenesis cannot be determined from this study. The present report is the first to demonstrate that voluntary running increases the survival of newly born cells and neurons in the DG of adolescent mice.

In the absence of TLX, a key regulator of adult neurogenesis, we found no pro-neurogenic effect of exercise. It has been previously shown that deletion of TLX leads to significant reduction in adult neurogenesis, synaptic plasticity, and impaired dendritic structure in the DG of adult mice (Christie et al., 2006). Similarly, we show reduced survival of newborn cells and neurons in the DG of sedentary Nr2e1$^{-/-}$ adolescent mice, an effect which was not mitigated by exercise. This positions TLX as a regulator of exercise-induced increases in neurogenesis during adolescence. Further studies will determine whether TLX mitigates the pro-neurogenic effects of exercise in adulthood and indeed throughout the lifespan. We have previously shown that a lack of TLX is associated with impairments in hippocampal-related cognitive and anxiety behaviors during adolescence. Specifically, adolescent but not adult Nr2e1$^{-/-}$ mice showed deficits in spatial working memory, contextual fear conditioning and cued fear conditioning (O'Leary, Kozareva, et al., 2016). The fact that a lack of TLX expression from birth through adulthood did not persistently induce the same impairments during adolescence and adulthood may point toward compensatory mechanisms occurring past the adolescent period, which

ameliorate to some degree the deficits caused by deletion of TLX, independent of neurogenesis. It is worth noting that in contrast to our data with TLX deficiency, ablation of neurogenesis through irradiation, another model of reduced hippocampal neurogenesis, has been shown to be sensitive to the rescue effects of exercise (Clark et al., 2008; Naylor et al., 2008). However, the effects of adolescent hippocampal irradiation on neuronal survival remain unknown.

We observed an exercise-induced increase in neurogenesis, but not cell survival, in the DG of single-housed adolescent mice which is consistent with previous reports using adult mice (Dostes et al., 2016; Gregoire et al., 2014; Kannangara, Webber, Gil-Mohapel, & Christie, 2009). It is worth noting however that single housing has been shown to blunt cell proliferation in running rats (Leasure & Decker, 2009; Stranahan, Khalil, & Gould, 2006) but these differences have not been reconciled in adolescent rodents. To date, the effect of exercise and housing conditions on neurogenesis across the septotemporal axis of the hippocampus has not been examined. This is interesting in light of the emerging view that the ventral hippocampus may be the predominant sub-region involved in stress responses (Bannerman et al., 2004; O'Leary & Cryan, 2014; Tanti & Belzung, 2013). Hence, the conflict in findings on hippocampal neurogenesis from group-housed and single-housed rodents may be a function of the differential effects of exercise on the dorsal and ventral hippocampus. Our results demonstrate that social isolation prevents the pro-neurogenic effects of exercise in the vDG but not the dDG. Specifically, exercise increased the survival of new neurons in the dDG of both single- and pair-housed mice, but only in the vDG of pair-housed mice. In addition, the exercise-induced increase in neurogenesis in the dDG of both single and pair-housed mice was significantly attenuated by single housing. Importantly, the studies to date report on exercise-induced increases in neurogenesis in the whole DG, which may explain why studies using single-housed mice consistently replicate the pro-neurogenic effects of exercise (Mustroph et al., 2012) observed in group-housed mice (van Praag, Christie, et al., 1999). Here, we show that social isolation during adolescence acts as differential regulator of exercise across the distinct anatomical regions of the DG and propose that neurogenesis in both the dDG and vDG should be taken into consideration when investigating the role of hippocampal neurogenesis in exercise and stress-induced changes in behavior. Nonetheless, the effects reported here may be specific to the adolescent period. Thus, an examination of the effect of social isolation stress during other time periods of the lifespan on any potential exercise-induced changes in neurogenesis in the subregions of the DG is warranted in future studies.

The mechanisms underlying the differential effect of social isolation stress on exercise-induced changes in neurogenesis in the dDG and vDG during adolescence remain unclear. It has been reported that unpredictable chronic mild stress in adult mice preferentially decreased the survival of new neurons in the vDG (Elizalde et al., 2010; Tanti, Rainer, Minier, Surget, & Belzung, 2012), supporting the view that neurogenesis in the ventral pole of the DG may be more susceptible to the effects of stress. Interestingly, environmental enrichment, which includes exercise, promoted neurogenesis only in the dorsal hippocampus (Tanti et al., 2012). The effects of stress on neurogenesis are thought to be mediated by the glucocorticoid (GR) and mineralocorticoid (MR) receptors (Saaltink & Vreugdenhil, 2014), which are highly expressed in the hippocampus of rodents (Montaron et al., 2003). However, there are some preliminary and inconclusive findings regarding the difference in expression levels of the GR receptor in the dDG and vDG (Lin, Lin, & Wang, 2012; Robertson, Beattie, Reid, & Balfour, 2005), while MR receptor expression has been shown to be more concentrated in the vDG, at least in the rat brain (Robertson et al., 2005). Notwithstanding, both receptors have been shown to have a distinct activation pattern across the septotemporal axis of the DG in response to acute stress (Caudal, Jay, & Godsil, 2014; Dorey, Pierard, Chauveau, David, & Beracochea, 2012). Moreover, a recent report has demonstrated that exercise increased GR expression in the hippocampus in single-housed but not pair-housed adult mice (Pan-Vazquez et al., 2015). Whether adolescent social isolation stress can affect the pro-neurogenic effect of exercise through the differential expression and activation of GR and MR in the vDG remains to be investigated. Another potential vDG-mediated mechanism underlying the attenuation of exercise-induced increase in neurogenesis by stress is through changes in the pro-neurogenic plasticity molecule BDNF (Berchtold, Chinn, Chou, Kesslak, & Cotman, 2005; Chen & Russo-Neustadt, 2009; Ploughman et al., 2007; Tang, Chu, Hui, Helmeste, & Law, 2008). This is due to the fact that a stressful spatial navigation task has previously been shown to differentially affect the expression of BDNF in the dorsal (increased expression) and ventral (decreased expression) subregions of the DG (Hawley, Morch, Christie, & Leasure, 2012). The *N*-methyl-D-aspartate (NMDA) receptor activation pathway has also been implicated in both stress- and exercise-related changes in hippocampal neurogenesis. Specifically, exercise induced a robust increase in the activation of NMDA receptor, albeit in cortical mouse tissue (Dietrich et al., 2005), and NMDA receptors have been reported to operate downstream of the stress hormone, corticosterone to regulate hippocampal neurogenesis (Cameron, Tanapat, & Gould, 1998). Given that there is a lower density of binding sites for NMDA receptors in the vDG compared to dDG (Pandis et al., 2006), it could be speculated that the two stimuli (isolation stress and exercise) compete for activation of the same pathway. Finally, isolation stress and exercise may also differentially impact on cell death in the dorsal and ventral DG. In the current study, we show a significant decrease in the number of apoptotic cells in the vDG of pair housed mice with access to a running wheel. This may reflect a protective mechanism of exercise against cell death, which is attenuated by isolation-induced stress. However, it is important to note that the apoptosis measured here accounted for all cells in the vDG; hence, we cannot conclude that the effects of isolation stress and exercise on apoptosis are specific to newly generated neurons.

It is surprising that we found no differences in the survival of newborn neurons of either area of the DG in single- and pair-housed sedentary adolescent mice. Studies conducted during adulthood have reported that social isolation results in anxiety- and depression-like behaviors in mice along with a reduction in levels of neuroplasticity genes (Berry et al., 2012; Ieraci, Mallei, & Popoli, 2016). Social isolation during adolescence in non-human primates (marmosets) also impaired hippocampal neurogenesis in time-dependent manner (Cinini et al.,

2014). Moreover, social isolation during adulthood has been shown to delay the pro-neurogenic effects of exercise in rats (Stranahan et al., 2006). Interestingly, evidence from the Pereira lab suggests that an enriched environment is necessary to promote neurogenesis in single-housed adult mice (Monteiro, Moreira, Massensini, Moraes, & Pereira, 2014). It is possible that in the absence of other external stimuli, social condition does not affect neurogenesis during the adolescent period, possibly due to the high basal rate of neurogenesis that occurs during adolescence compared to adulthood (He & Crews, 2007). Alternatively, the mouse strain used in the current study (generated on a BL6129 background) may have been a confounding factor by potentially limiting our ability to detect any downregulation of new neurons. In several independent studies examining the role of genetic influence on the baseline level of hippocampal neurogenesis, the B6129SF1 and 129Sv were among the strains showing the lowest levels of newborn neurons (Clark et al., 2011; Kempermann, Kuhn, & Gage, 1997; Merritt & Rhodes, 2015).

In conclusion, our results demonstrate that social isolation stress during adolescence attenuates an exercise-induced increase in neurogenesis. We show that this effect is most pronounced in the ventral hippocampus, a brain sub-region which plays a predominant role in anxiety and in regulating the stress response. Adolescence is a critical period for susceptibility to stress-related disorders as well as a time during which remodeling of hippocampal connectivity, including neurogenesis occurs. Thus, the impact of stress during adolescence on hippocampal neurogenesis and associated behaviors may be particularly potent. We also show that TLX is necessary for the pro-neurogenic effects of exercise during adolescence and have previously shown that the role of TLX in anxiety-related behaviors is most apparent during adolescence. TLX is thus an important intrinsic regulator of exercise-induced changes in neurogenesis and may be a key target in understanding the interaction between positive and negative modifiable lifestyle factors such as stress and exercise on hippocampal neurogenesis and associated behaviors during adolescence.

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ORCID

Yvonne M. Nolan http://orcid.org/0000-0003-2426-234X

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Research report

The nuclear receptor Tlx regulates motor, cognitive and anxiety-related behaviours during adolescence and adulthood

James D. O'Leary[a,1], Danka A. Kozareva[a,1], Cara M. Hueston[a], Olivia F. O'Leary[a,b], John F. Cryan[a,b], Yvonne M. Nolan[a,*]

[a] Department of Anatomy and Neuroscience, University College Cork, Ireland
[b] APC, Microbiome Institute, University College Cork, Ireland

HIGHLIGHTS

- The nuclear receptor Tlx regulates motor, cognitive and anxiety-related behaviours during adolescence and adulthood.
- The role of Tlx in spatial working memory is most apparent in adolescence.
- Regulation of anxiety-related behaviour by Tlx is sex-dependent.
- The effects of Tlx deletion on hyperactivity are sex-independent.
- Regulation of cortical-striatal behaviour by Tlx is age-dependent.

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ABSTRACT

The nuclear receptor Tlx is a key regulator of embryonic and adult hippocampal neurogenesis and has been genetically linked to bipolar disorder. Mice lacking Tlx (*Nr2e1*$^{-/-}$) display deficits in adult hippocampal neurogenesis and behavioural abnormalities. However, whether Tlx regulates behaviour during adolescence or in a sex-dependent manner remains unexplored. Therefore, we investigated the role of Tlx in a series of behavioural tasks in adolescent male and female mice with a spontaneous deletion of Tlx (*Nr2e1*$^{-/-}$ mice). Testing commenced at adolescence (postnatal day 28) and continued until adulthood (postnatal day 67). Adolescent male and female *Nr2e1*$^{-/-}$ mice were hyperactive in an open field, an effect that persisted in adulthood. Male but not female *Nr2e1*$^{-/-}$ mice exhibited reduced thigmotaxis during adolescence and adulthood. Impairments in rotarod motor performance developed in male and female *Nr2e1*$^{-/-}$ mice at the onset of adulthood. Spontaneous alternation in the Y-maze, a hippocampus-dependent task, was impaired in adolescent but not adult male and female *Nr2e1*$^{-/-}$ mice. Contextual fear conditioning was impaired in adolescent male *Nr2e1*$^{-/-}$ mice only, but both male and female adolescent *Nr2e1*$^{-/-}$ mice showed impaired cued fear conditioning, a hippocampal-amygdala dependent cognitive process. These deficits persisted into adulthood in males but not females. In conclusion, deletion of Tlx impairs motor, cognitive and anxiety-related behaviours during adolescence and adulthood in male and female mice with most effects occurring during adolescence rather than adulthood, independent of housing conditions. This suggests that Tlx has functions beyond regulation of adult hippocampal neurogenesis, and may be an important target in understanding neurobiological disorders.

1. Introduction

The orphan nuclear receptor Tlx, encoded by the gene *Nr2e1*, is a key regulator of embryonic and adult neurogenesis, with expression localized within the neurogenic niche of the forebrain and retina [1–3]. Tlx has been shown to be crucial for neural and retinal development [4,5]. Mice lacking Tlx display hypoplasia of the retina, cerebrum and olfactory bulbs as well as malformation of the limbic system, specifically the dentate gyrus within the hippocampus [5–9]. Moreover, deletion of Tlx has been shown to impair adult neurogenesis, synaptic plasticity and to negatively affect dendritic structure within the dentate gyrus of adult mice [10]. Thus, alterations in Tlx expression are likely to affect hippocampus-dependent behaviour.

* Corresponding author.
E-mail address: y.nolan@ucc.ie (Y.M. Nolan).
[1] Equal contribution.

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Several different mouse models have been developed to target Tlx *in vivo*, such as targeted disruption by homologous recombination [3,7], spontaneous deletion [8] and conditional deletion [11] (collectively referred to here as *Nr2e1*$^{-/-}$). Differences between these models make it difficult to draw comparisons. However, similarities are seen across models as mice with impairments in Tlx function have shown a number of behavioural abnormalities. The most striking behavioural phenotype of mice with a spontaneous deletion is aggression, which is regulated by the prefrontal cortex and limbic system [12]. This circuitry has previously been shown to be defective in *Nr2e1*$^{-/-}$ mice with both a targeted disruption of Tlx by homologous recombination and spontaneous deletion [6,8]. Hyperactivity has also been documented in mice with a spontaneous deletion of Tlx from as early as postnatal day (P) 18 [13]. Furthermore, impairments in spatial learning have been observed in adult mice with a conditional deletion of Tlx, while contextual and cued fear memory were unaffected [11]. Conversely, it has been shown that following a targeted disruption of Tlx by homologous recombination, mice exhibited poor contextual and cued fear recall, despite normal fear acquisition, in addition to reduced anxiety-like behaviour within the elevated plus maze [7]. The reasons for the discrepancies across studies in adult mice are unclear but may be a function of the different methods used to reduce or inhibit Tlx expression, and/or when Tlx disruption occurs, such as early life or adulthood [7,14]. When the Tlx transgene was overexpressed using lentivirus-mediated means or in transgenic mice, an increase in adult hippocampal neurogenesis and enhanced performance in the Morris water maze as well as prepulse inhibition was observed [15]. This work suggests a role for Tlx in learning and memory through its regulatory effect on adult hippocampal neurogenesis.

The mammalian brain continues to develop after birth, throughout childhood and into adulthood [16,17]. The adolescent period, which occurs in mice in postnatal weeks 3–8 [18], is a critical developmental window when crucial neural circuits are established via a period of synaptic re-modelling [19,20] and is a key period for susceptibility to stress and the emergence of neurobiological disorders such as schizophrenia, depression and anxiety [21,22] [21–25]. Interestingly, linkage analysis studies of patients with bipolar disorder have reported susceptibility loci on chromosomes where *Nr2e1* is expressed [26] thus suggesting a potential link between Tlx and mood disorders. Several studies have characterized the expression and the functional role of Tlx within the brain during embryonic and early postnatal development [1,2,7,13,27]. However, the functional role of Tlx during adolescence remains largely unexplored. In particular, it is not yet clear whether there are critical periods during postnatal life when Tlx might play a more dominant role in cognition, and whether such effects are sex-dependent. Thus, the aim of this study was to explore the extent and involvement of Tlx in hippocampus- dependent cognition as well as hippocampus-independent functions during adolescence and adulthood in both male and female mice.

2. Materials and method

2.1. Experimental design

Behavioural analysis was carried out in male and female mice with a spontaneous deletion of the Tlx gene (*Nr2e1*$^{-/-}$), heterozygous (*Nr2e1*$^{+/-}$) and wild type littermates. In order to capture potential deficits that may manifest during the adolescent developmental period, behavioural testing commenced at P28 and continued into adulthood until P67. Sensorimotor tests and motor performance tests on the rotarod were conducted each week. Open field tests, spontaneous alternation in the Y-maze, and contextual and cued fear conditioning were conducted during adolescence (P28-35), and again in adulthood (P56-67; see Fig. 1 for experimental design). *Nr2e1*$^{-/-}$ mice display impaired eye sight, therefore the behavioural tasks employed were chosen to minimize the dependency on visuospatial learning as much as possible [28–30].

2.2. Animals

The animals used in the present study were all first generation offspring on a hybrid B6129 background resulting from mating male heterozygote (*Nr2e1*$^{+/-}$) mice on a 129S1/SvImJ background with female heterozygote (*Nr2e1*$^{+/-}$) mice on a C57BL/6J background. They were kindly provided by Prof. Elizabeth Simpson, University of British Colombia and were generated as previously described [13]. These mice exhibit a spontaneous deletion of the entire *Nr2e1* allele, including all nine exons. However, the deletion of the Tlx gene does not affect the transcription of neighbouring genes [31]. The impact of maternal care was controlled for as all animals were first generation littermate offspring resulting from mating male heterozygote (*Nr2e1*$^{+/-}$) mice with female heterozygote (*Nr2e1*$^{+/-}$) mice. All pups were weaned at P21. Due to the aggression that has been previously described in this strain, male *Nr2e1*$^{-/-}$ mice were singly housed after weaning [8]. Male wild type and heterozygous littermates and all female mice were grouped housed in standard housing conditions (temperature 21 °C and relative humidity 55%). All mice had food and water available *ad libitum*. All experiments were conducted in accordance with the European Directive 2010/63/EU, and under an authorization issued by the Health Products Regulatory Authority Ireland and approved by the Animal Ethics Committee of University College Cork.

2.3. Body weight, growth rate and primary observation tests

Animals were weighed and growth rate calculated each week [(present weight − past weight)/past weight x100]. Sensorimotor tests were conducted to identify any gross impairment which may have affected behavioural testing. The primary observation scores and sensorimotor testing were adapted from the Irwin behavioural screen [32–34]. This included measures of general health and physical appearance as well as sensorimotor reflexes, piloerection, palpebral closure, salivation, tremors, gait, trunk curl, pinna reflex, whisker reflex, reaching reflex, eye reflex, righting reflex, toe pinch, and provoked biting as a measure of aggression. Observations were recorded each week (P30, P37, P44, P51 and P58) and a score was assigned as indicated in Table 1.

2.4. Locomotor activity and thigmotaxis in the open field

Spontaneous exploratory locomotor activity and thigmotaxis in the open field were used as a general measure of motor function and anxiety-related behaviours, respectively [32]. Animals were placed within a rectangular open field (32 × 40 cm; made in house) for 10 min. Locomotor activity is a simple measure of the distance the animal travels within the open field during the test, where large distances indicate hyperactivity. Thigmotaxis refers to the tendency of rodents to stay close to the walls of a maze during exploration [32,35]. The behavioural test measures anxiogenesis induced by exposure to a novel environment as rodents tend to avoid open spaces and stay close to borders of maze arenas. Both locomotor activity and thigmotaxis were analysed using specialized software (Ethovision XT, Noldus Information Technology, USA).

2.5. Motor performance in the rotarod

Performance on the accelerating rotarod is a well-established measure of motor performance [32] and was assessed in this

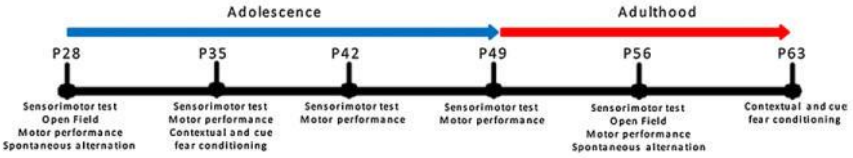

Fig. 1. Experimental design. *Nr2e1*$^{-/-}$, *Nr2e1*$^{+/-}$ and wild type mice were tested during adolescent development (postnatal day 28–49) and adulthood (postnatal day 56–67).

Table 1
Primary observation of Nr2e1$^{-/-}$ mice.

Physical Characteristics				Sensorimotor Reflexes			
Presence of Whiskers	Score	Appearance of Fur	Score	Gait	Score	Trunk Curl	Score
None	0	Ungroomed and disheveled	0	Normal	0	Absent	0
A few	1	Somewhat disheveled	1	Fluid but abnormal	1	Present	1
Most, but not a full set	2	Well-groomed (normal)	2	Limited movement only	2		
A full set	3			Incapacity	3	Eye Reflex	
		Wounds				Present	0
Piloerection		None	0	Reaching Reflex		Absent	1
None	0	Signs of previous wound	1	None	0		
Most hairs on end	1	Slight wounds present	2	Upon nose contact	1	Whisker Reflex	
		Moderate wounds present	3	Upon vibrissae contact	2	Present	0
Respiration		Extensive wounds present	4	Before vibrissae contact	3	Absent	1
Gasping, irregular	0			Early vigorous extension	4		
Slow, shallow	1	Salivation				Toe Pinch	
Normal	2	None	0	Provoked Biting		None	0
Hyperventilation	3	Slight margin of sub-maxillary area	1	Absent	0	Slight withdrawal	1
		Wet zone entire sub-maxillary area	2	Present	1	Moderate withdrawal, not brisk	2
Patches of Fur missing on Face						Brisk, rapid withdrawal	3
None	0	Patches of Fur missing on body		Pinna Reflex		Very brisk, repeated	4
Some	1	None	0	None	0	extension and flexion	
Extensive	2	Some	1	Active retraction, moderately brisk flick	1		
		Extensive	2	Hyperactive, repetitive flick	2		
Palpebral Closure							
Eyes wide open	0	Skin Color		Righting Reflex		Tremor	
Eyes 1/2 closed	1	Blanched	0	No impairment	0	None	0
Eyes closed	2	Pink	1	Number of sec required to right	1 to 10	Mild	1
		Bright, deep red, flushed	2			Marked	2

paradigm using a protocol adapted from Menalled, El-Khodor [36]. The mice were placed on the rotarod apparatus (Ugo Basile, Italy) for five minutes and tested on an accelerating protocol (4 RPM to 40 RPM over five minutes, averaging 7.2 RPM acceleration). The latency for each mouse to fall was recorded. The mice were tested during three trials a day for three consecutive days (total nine trials), with the best score recorded. The test was repeated at weekly intervals beginning P28, P35, P42, P49 and P56, respectively. A reduced latency to fall indicates impairment in motor performance and suggests a dysfunction within the cortical-striatal circuit which regulates motor behaviour.

2.6. Spontaneous alternation in the Y maze

Spontaneous alternation behaviour is the tendency of rodents to alternate their exploration of maze arms (such as those of the Y maze) and is used as a measure of hippocampal-dependent working memory as previously described [37]. The Y maze consisted of three arms 120° from each other (16 cm × 6.5 cm; made in house). The protocol was adapted from Senechal et al. [38]. Each animal was placed into the first arm of the maze facing the wall, and allowed to explore the maze for five minutes. The number and order of arm entries were recorded. An arm entry was defined as all four paws entering into the arm (four paw criteria). An alternation was determined as the number of consecutive entries into the three maze arms. Alternations were then divided by the total number of entries during the five minute test period.

2.7. Contextual and cued fear conditioning

Contextual fear conditioning was used to assess hippocampal-dependent learning, while cued fear conditioning was employed to probe amygdala-dependent cognitive processes as previously described [39,40]. During acquisition, animals were first placed into the fear conditioning chamber (Med Associates, 30.5 cm × 24.1 cm × 21.0 cm) which was scented with a lemon and ginger tea bag (Twinings^{TM}). Animals were allowed to explore the chamber for two minutes during an acclimation period and then received three shock and tone pairs (30 s tone; 5 kHz; 70 dB; 1 s foot shock; 0.65 mA DC current) separated by 30 s intervals. Animals were placed back in their home cage one minute after the final shock. Contextual fear memory was assessed 24 h later by placing the animals back into the same chamber, but in the absence of tone and shock. Freezing behaviour (sec) was measured during the last 3.5 min of the total 5.5 min protocol using specialized software (Video freeze, Med Associates, USA).

Cued fear conditioning was measured 24 h after the contextual test in the same chambers. To measure cued fear learning, animals were placed into a novel context (white floor; black wall insert at 60°; and almond scent 1%) with presentation of the tone but no foot shock. Animals were allowed two minutes to acclimatize followed by three tone presentations (30 s; 5 kHz; 70 dB). Freezing behaviour during the 30 s tone presentations was recorded (Video freeze, Med Associates, USA). Contextual and cued fear conditioning was assessed during adolescence and adulthood with mice reconditioned to the tone and context in adulthood. Prior to reconditioning

in adulthood, mice were placed back into the initial shock chamber to assess contextual fear memory retention. Twenty four hours later mice were placed in the same chamber as the cued fear conditioning chamber in order to assess cued fear memory recall retention.

2.8. Statistical analysis

All data were analysed using SPSS statistical software (SPSS, Chicago, IL). Data from body weight, rotarod motor performance, and cued fear conditioning were analysed by repeated measures ANOVA with Bonferroni post hoc test. Data from open field, spontaneous alternation and contextual fear conditioning were analysed by one-way ANOVA, with Fisher's LSD post hoc analysis. Non-parametric data from sensorimotor tests were analysed by the Kruskal–Wallis one-way ANOVA. An alpha level of 0.05 was used as criterion for statistical significance. Parametric data are presented as mean ± SEM. Nonparametric data are presented as percentage (%) displaying normal response.

3. Results

3.1. Nr2e1$^{-/-}$ mice have reduced body weight and increased growth rate during adolescence

Male and female mice gained weight throughout development (Fig. 2a and b). The Mauchly's test indicated that in the male cohort the assumptions of sphericity had been violated (χ^2 (9) = 51.29, $p < 0.01$). Therefore the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity as the epsilon was less than 0.75 ($\varepsilon = 0.595$). The results show that all male mice gain body weight with age (F (2.38, 83.32) = 421.39, $p < 0.01$; Fig. 2a). Female mice also showed a similar result, with all genotypes gaining weight with age (F (4, 136) = 253.83, $p < 0.01$; Fig. 2b). There was a significant effect of genotype on body weight throughout adolescence and adulthood, in both male (F (2, 35) = 17.74, $p < 0.01$) and female (F (2, 34) = 20.35, $p < 0.01$) mice, with *Nr2e1*$^{-/-}$ mice remaining significantly lighter than their wild type and heterozygous littermates (Bonferroni post hoc comparison, $p < 0.01$). There was also a significant interaction between age and genotype in male (F (4.76, 83.32) = 4.40, $p < 0.01$) and female (F (8, 136) = 3.21, $p < 0.01$) mice, indicating that *Nr2e1*$^{-/-}$ mice do not gain weight similarly to their wild type and *Nr2e1*$^{+/-}$ littermates.

Male and female mice show a reduction in the rate of growth as they approach adulthood (Fig. 2c and d). Interestingly, *Nr2e1*$^{-/-}$ mice appear to have a higher rate of growth during early adolescence (P35) compared to wild type and *Nr2e1*$^{+/-}$ littermates in both males (F (2, 35) = 18.69, $p < 0.01$) and females (F (2, 34) = 24.66, $p < 0.01$). Furthermore, this increased growth rate appears to normalize at the onset of adulthood (P49-56). These results indicate that while *Nr2e1*$^{-/-}$ mice gain body weight at a greater rate throughout development, body weight remains reduced compared to wild type and heterozygous littermates. This finding is stable across sex and is independent of housing conditions.

3.2. Nr2e1$^{-/-}$ mice exhibit increased provoked biting and impaired eye reflex, reaching reflex and piloerection in a primary observation test battery

The results of the primary observation tests are summarised in Tables 2 and 3. There was no significant difference across genotype, sex or age in animal appearance (presence of whiskers, appearance of fur, and patches of fur missing, skin colour), respiration, tremors, salivation, gait, trunk curl, pinna reflex, whisker reflex, toe pinch reflex or righting reflex. However, both male and female *Nr2e1*$^{-/-}$ mice showed an increase in provoked biting during adolescence (male, $p < 0.05$ and female, $p < 0.05$; Table 2). This increase in provoked biting continued in adulthood (Table 3) but did not reach statistical significance (male, $p = 0.14$ and female, $p = 0.13$). Male *Nr2e1*$^{-/-}$ mice also exhibited impaired eye reflex during adolescence ($p < 0.05$; Table 2). However, this was not observed during adulthood ($p > 0.05$; Table 3). In addition, adolescent male *Nr2e1*$^{-/-}$ mice showed a trend towards impaired reaching reflex ($p = 0.057$; Table 2). This pattern continued during adulthood in male mice but did not reach statistical significance (males $p = 0.12$; females $p > 0.05$; Table 3). Further, adult male *Nr2e1*$^{-/-}$ mice exhibited an increase in piloerection ($p < 0.01$) and an increase in palpebral closure ($p < 0.05$; Table 3).

3.3. Nr2e1$^{-/-}$ mice exhibit hyperactivity and deficits in cortico-striatal associated behaviour

3.3.1. Locomotor activity in the open field

Testing in the open field revealed that male (Fig. 3a) and female (Fig. 3b) *Nr2e1*$^{-/-}$ mice were hyperactive during adolescence (male F (2, 37) = 25.21, $p < 0.01$, female F (2, 36) = 18.05, $p < 0.01$), independent of housing conditions. Hyperactivity continued into adulthood in both male (F (2, 37) = 37.79, $p < 0.01$) and female (F (2, 36) 19.77, $p < 0.01$) *Nr2e1*$^{-/-}$ mice (Fig. 3c and d, respectively). Furthermore, hyperactivity appeared to be more pronounced during adulthood in both male and female *Nr2e1*$^{-/-}$ mice with an approximately three-fold increase in distance travelled compared to wild types (Fig. 3c and d).

3.3.2. Thigmotaxis in the open field

Adolescent male *Nr2e1*$^{-/-}$ mice exhibited a significant increase in exploration of the centre of the open field (F (2, 37) = 3.90, $p < 0.05$) indicating a reduction in thigmotaxis behaviour (Fig. 3e). This observation continued into adulthood (F (2, 37) = 6.37, $p < 0.01$; Fig. 3 g). All female mice showed similar thigmotaxis behaviour throughout adolescence (F (2, 36) = 0.46, $p > 0.05$) and adulthood (F (2, 36) = 1.39, $p > 0.05$; Fig. 3f and h, respectively).

3.3.3. Motor performance on the rotarod

The Mauchly's test indicated the assumptions of sphericity had been violated in both male (χ^2 (9) = 27.64, $p < 0.01$) and female (χ^2 (9) = 18.77, $p < 0.027$) mice. Therefore, the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity for males, as the epsilon was less than 0.75 ($\varepsilon = 0.74$), and the Huynh-Feidt estimates of sphericity for females, as the epsilon was greater than 0.75 ($\varepsilon = 0.949$). With this correction, testing on the rotarod revealed that impairments in motor performance developed at the onset of adulthood (P42; Fig. 4a and b) in both male (F (2.69, 103.603) = 4.54, $p < 0.01$) and female (F (3.795, 129.026) = 9.36, $p < 0.01$) Nr2e1$^{-/-}$ mice, independent of housing conditions. There was a significant effect of genotype on motor performance, in both male (F (2, 35) = 6.88, $p < 0.01$; Fig. 4a) and female (F (2, 34) = 7.59, $p < 0.01$; Fig. 4b) Nr2e1$^{-/-}$ mice. There was also a significant interaction between age and genotype in male (F (5.92, 103.603) = 3.344, $p < 0.01$) and female (F (7.59, 109.202) = 2.97, $p < 0.01$) mice. Bonferroni post hoc comparison revealed that impairments in motor performance developed in both male and female Nr2e1$^{-/-}$ mice at the onset of adulthood (Fig. 4a and b). This indicates that motor performance does not remain stable throughout development for Nr2e1$^{-/-}$ mice.

3.4. Nr2e1$^{-/-}$ mice exhibit deficits in hippocampus-associated cognition

3.4.1. Spontaneous alternation in the Y maze

In the spontaneous alternation test of working memory, male and female *Nr2e1*$^{-/-}$ mice showed impaired spontaneous alternation during adolescence compared to *Nr2e1*$^{+/-}$ and wild type mice

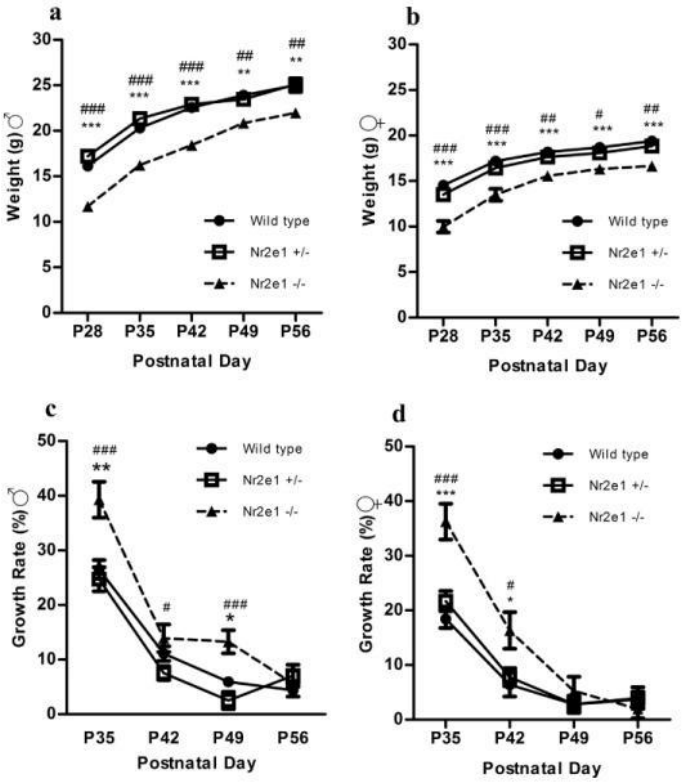

Fig. 2. Body weight as a function of genotype, sex and age. Body weight of male (a) and female (b) mice; growth rate of male (c) and female (d) mice. ***p < 0.0001, **p < 0.001, *p < 0.05, *Nr2e1*$^{-/-}$ compared to wild type mice. ###p < 0.0001, ##p < 0.001, #p < 0.05, *Nr2e1*$^{-/-}$ compared to *Nr2e1*$^{+/-}$ mice; ANOVA with post hoc Bonferroni analysis. All results are expressed as mean ± SEM. Sample size per sex: wild type (n = 13–14), *Nr2e1*$^{+/-}$ (n = 16), *Nr2e1*$^{-/-}$ (n = 8).

Table 2
Primary Observation in adolescent mice.

Male				Female			
Physical Characteristics	Wild type	Nr2e1$^{+/-}$	Nr2e1$^{-/-}$	Physical Characteristics	Wild type	Nr2e1$^{+/-}$	Nr2e1$^{-/-}$
Presence of Whiskers (%)	100	100	100	Presence of Whiskers (%)	100	100	100
Well-groomed fur (%)	100	100	100	Well-groomed fur (%)	100	100	100
Piloerection (%)	0	0	0	Piloerection (%)	0	0	0
Missing fur on Face (%)	21.4	13.3	0	Missing fur on Face (%)	0	12.5	12.5
Missing fur on body (%)	0	0	12.5	Missing fur on body (%)	7.6	18.7	12.5
Palpebral Closure (%)	0	0	0	Palpebral Closure (%)	0	0	0
Wounds (%)	0	6.25	0	Wounds (%)	0	6.2	0
Respiration	Normal	Normal	Normal	Respiration	Normal	Normal	Normal
Tremor (%)	0	0	0	Tremor (%)	0	0	0
Skin Color	Normal	Normal	Normal	Skin Color	Normal	Normal	Normal
Salivation (%)	0	0	0	Salivation (%)	0	0	0
Sensorimotor Reflexes (% displaying normal response)				Sensorimotor Reflexes (% displaying normal response)			
Gait	100	100	100	Gait	100	100	100
Trunk Curl	100	100	100	Trunk Curl	92.3	93.7	100
Reaching Reflex	71.4	100	62.5	Reaching Reflex	92.3	87.5	62.5
Pinna Reflex	85.7	87.5	75	Pinna Reflex	100	75	87.5
Eye Reflex	85.7	100	62.5*	Eye Reflex	90.09	66.67	100
Whisker Reflex	85.7	92.85	75	Whisker Reflex	100	80	100
Toe Pinch	78.5	81.25	62.25	Toe Pinch	100	81.25	100
Righting Reflex (% impaired)	0	0	0	Righting Reflex (% impaired)	0	0	0
Provoked Biting (%)	38.4	31.2	87.5*	Provoked Biting (%)	30.7	37.5	77.8*

Nr2e1$^{-/-}$ compared to wild type mice. Kruskal–Wallis one-way ANOVA. Sample size per sex: wild type (n = 13–14), *Nr2e1*$^{+/-}$ (n = 16), *Nr2e1*$^{-/-}$ (n = 8).
* p < 0.05.

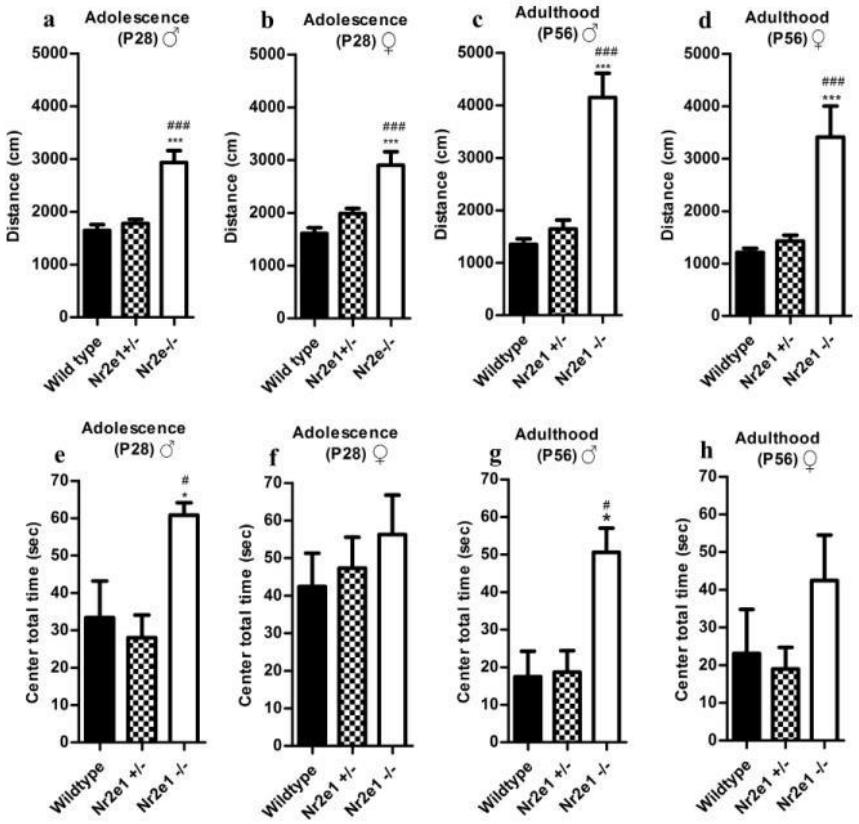

Fig. 3. Locomotor activity and thigmotaxis in an open field as a function of genotype. Locomotor activity in adolescent male (a) and female (b) mice, and in adult male (c) and female (d) mice. Exploration of arena centre in adolescent male (e) and female (f) mice, and in adult male (g) and female (h) mice. ***p < 0.0001, **p <0.001, *p < 0.05, *Nr2e1*$^{-/-}$ compared to wild type mice. ###p < 0.0001, ##p < 0.001, #p < 0.05, *Nr2e1*$^{-/-}$ compared to *Nr2e1*$^{+/-}$ mice; ANOVA with post hoc Bonferroni analysis. All results are expressed in mean ± SEM. Sample size per sex: wild type (n = 13–14), *Nr2e1*$^{+/-}$ (n = 16), *Nr2e1*$^{-/-}$ (n = 8).

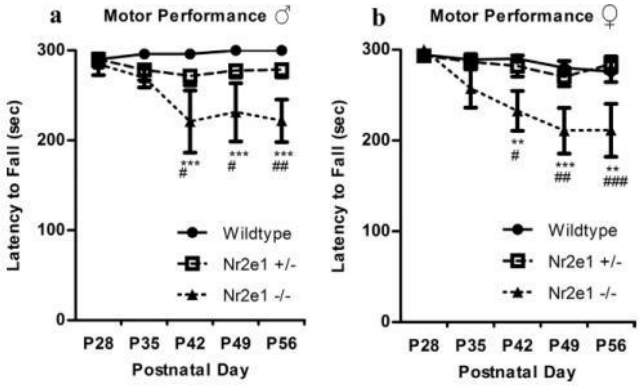

Fig. 4. Motor performance in a Rota-rod latency to fall paradigm as a function of genotype. Motor performance on the Rotarod in male (a) and female (b) mice. ***p < 0.0001, **p < 0.001, *p < 0.05, *Nr2e1*$^{-/-}$ compared to wild type mice. ### p < 0.0001, ##p < 0.001, #p < 0.05, *Nr2e1*$^{-/-}$ compared to *Nr2e1*$^{+/-}$ mice; ANOVA with post hoc Bonferroni analysis. All results are expressed in mean ± SEM. Sample size per sex: wild type (n = 13–14), *Nr2e1*$^{+/-}$ (n = 16), *Nr2e1*$^{-/-}$ (n = 8).

Table 3
Primary observation in adult mice.

Male				Female			
Physical Characteristics	Wild type	Nr2e1$^{+/-}$	Nr2e1$^{-/-}$	Physical Characteristics	Wild type	Nr2e1$^{+/-}$	Nr2e1$^{-/-}$
Presence of Whiskers (%)	100	100	100	Presence of Whiskers (%)	84.6	81.2	87.5
Well-groomed fur (%)	76.9	75	62.5	Well-groomed fur (%)	100	93.7	100
Piloerection (%)	0	0	62.5***	Piloerection (%)	0	0	0
Missing fur on Face (%)	0	0	0	Missing fur on Face (%)	0	0	0
Missing fur on body (%)	0	0	0	Missing fur on body (%)	0	0	0
Palpebral Closure (%)	0	0	25*	Palpebral Closure (%)	0	0	12.5
Wounds (%)	7.7	12.5	37.5	Wounds (%)	0	0	12.5
Respiration	Normal	Normal	Normal	Respiration	Normal	Normal	Normal
Tremor (%)	0	0	0	Tremor (%)	0	0	0
Skin Color	100	100	100	Skin Color	100	100	100
Salivation (%)	7.7	0	33.33	Salivation (%)	0	13.3	0
–							
Sensorimotor Reflexes (% displaying normal response)				Sensorimotor Reflexes (% displaying normal response)			
Gait	100	100	100	Gait	100	100	100
Trunk Curl	100	100	100	Trunk Curl	84.6	93.7	100
Reaching Reflex	92.3	62.5	50	Reaching Reflex	92.3	87.5	62.5
Pinna Reflex	76.9	75	75	Pinna Reflex	100	62.5	87.5
Eye Reflex	92.3	81.25	100	Eye Reflex	100	86.66	83.33
Whisker Reflex	84.61	87.5	100	Whisker Reflex	77.77	86.66	100
Toe Pinch	100	93.7	100	Toe Pinch	92.3	93.7	100
Righting Reflex (% impaired)	0	0	0	Righting Reflex (% impaired)	0	0	0
Provoked Biting (%)	46.1	50	87.5	Provoked Biting (%)	46.1	31.2	75

Nr2e1$^{-/-}$ compared to wild type mice. Kruskal–Wallis one-way ANOVA. Sample size per sex: wild type (n = 13), *Nr2e1*$^{+/-}$ (n = 16), *Nr2e1*$^{-/-}$ (n = 8).
* $p < 0.05$.
*** $p < 0.0001$.

(male F (2, 37) = 4.60, $p < 0.01$ and female F (2, 36) = 3.97, $p < 0.05$; Fig. 5a and b). However, this effect did not persist into adulthood (Fig. 5c and d). A significant effect was observed in spontaneous alternation in adult male mice (F (2, 37) = 7.31, $p < 0.01$; Fig. 5c). Post hoc comparison using the Fisher's LSD test revealed this was due to an increase in *Nr2e1*$^{+/-}$ performance compared to wild type ($p < 0.01$) and *Nr2e1*$^{-/-}$ mice ($p < 0.01$). Female mice exhibited no overall difference in spontaneous alternation during adulthood (F (2, 36) = 1.80, $p > 0.05$; Fig. 5d).

3.4.2. Contextual fear conditioning

Male *Nr2e1*$^{-/-}$ mice showed impaired freezing behaviour during adolescence compared to *Nr2e1*$^{+/-}$ and wild type mice (F (2, 36) = 8.82, $p < 0.01$; Fig. 5e). Adolescent female *Nr2e1*$^{-/-}$ mice showed a trend for reduced contextual freezing but this did not reach statistical significance (F (2, 34) = 2.46, $p = 0.10$; Fig. 5f). Contextual freezing during adulthood did not differ across sex or genotype (male F (2, 36) = 2.34, $p > 0.05$; Fig. 5 g); female (F (2, 28) = 0.47, $p > 0.05$; Fig. 5 h).

3.5. *Nr2e1*$^{-/-}$ mice show impaired hippocampal-amygdala dependent cognition

3.5.1. Cued fear conditioning

Both male and female *Nr2e1*$^{-/-}$ mice showed impaired cued fear recall during adolescence (male F (2, 34) = 3.62, $p < 0.05$ and female F (2, 32) = 10.17, $p < 0.01$; Fig. 6a and d). In adulthood, only male *Nr2e1*$^{-/-}$ mice exhibited impaired cued fear recall (F (2, 34) = 14.31, $p < 0.01$; Fig. 6b). Interestingly, male *Nr2e1*$^{+/-}$ mice also exhibited impaired cued fear recall during adulthood, but not during adolescence. However, no impairment was observed in female *Nr2e1*$^{+/-}$ or *Nr2e1*$^{-/-}$ mice during adulthood (F (2, 26), = 0.96, $p > 0.05$; Fig. 6e).

To measure the retention of the cued fear memory that was acquired during adolescence, we first measured freezing behaviour in response to the cue but prior to the re-introduction of the unconditioned stimulus at P62 (Fig. 6c and f). In this cued fear memory retention test both male and female adult (P62) *Nr2e1*$^{-/-}$ mice exhibited poor retention of the fear memory that was acquired in adolescence (male F (2, 34) = 19.95, $p < 0.01$ and female F (2, 29) = 17.0, $p < 0.01$).

4. Discussion

This study demonstrated that Tlx has a role to play in motor, cognitive and anxiety-related behaviour during adolescence and adulthood independent of sex or housing conditions, with most impact during adolescence. Both adolescent male and female *Nr2e1*$^{-/-}$ mice showed deficits in spatial working memory as measured by spontaneous alternation in the Y maze. Further, adolescent male but not female *Nr2e1*$^{-/-}$ mice showed deficits in hippocampal function as measured by contextual fear conditioning but these effects in hippocampus-dependent memory tasks did not persist into adulthood. Previous studies have reported contradictory findings regarding the involvement of Tlx in hippocampus-associated cognition in adult mice. Similar to the present study, it has been shown that normal fear acquisition and contextual fear conditioning occurs in adult male *Nr2e1*$^{-/-}$ mice [11]. Impaired associative fear memory in contextual fear conditioning in adult male mice with a targeted disruption of Tlx has also been reported [7]. The present study is to our knowledge the first report of impaired spatial working memory in adolescent male and female *Nr2e1*$^{-/-}$ mice with a spontaneous deletion; however, this deficit did not persist into adulthood. This is in contrast to the previously reported impairments in spatial working memory in Tlx deficit mice albeit using the Morris water maze and in mice with a conditional deletion in adulthood [11], rather than a spontaneous deletion. The reasons for the discrepancies across studies in adult mice are unclear but may be a function of the different methods used to reduce or inhibit Tlx expression, or the time at which the Tlx disruption occurs. Zhang et al. generated a conditional deletion of Tlx in adult mice localized to the forebrain and olfactory bulbs, whereas Roy et al. generated a transgenic strain with a targeted disruption of Tlx [7,11]. In the mice used in the present study, the Tlx deletion occurs from birth via a spontaneous deletion of all nine exons of the gene. It is important to consider that germline mutation models such as the *Nr2e1*$^{-/-}$ mice used in the current study and by others (where Tlx disrup-

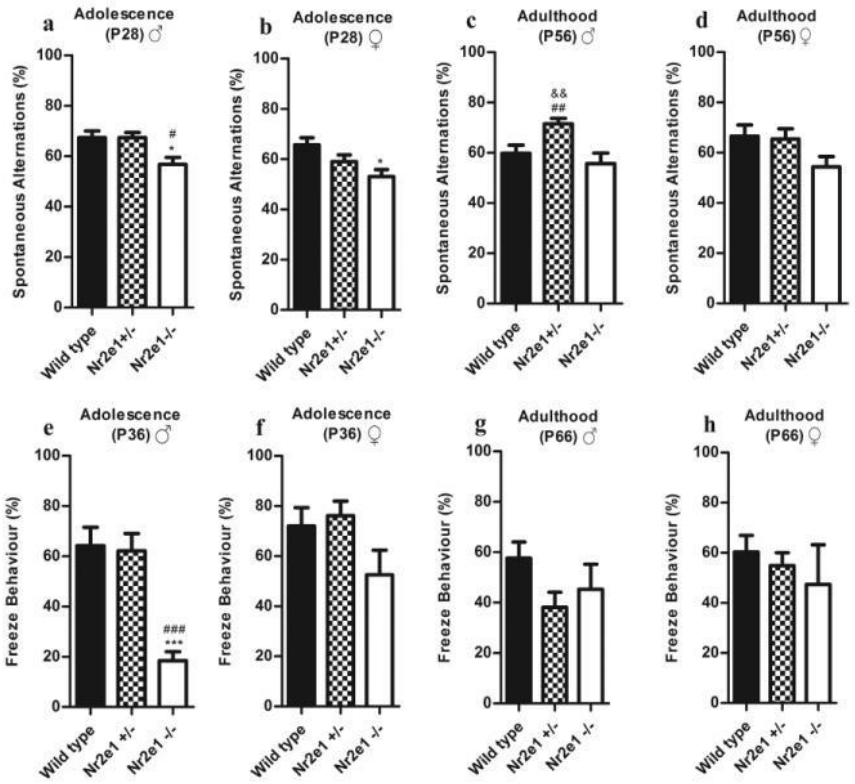

Fig. 5. Spontaneous alternation (%) in the Y-maze and contextual fear conditioning as a function of genotype during adolescence (P28-36) and adulthood (P56-66). Spontaneous alternation in adolescent male (a) and female (b) mice, and in adult male (c) and female (d) mice. Contextual freeze behaviour in adolescent male (e) and female (f) mice, and in adult male (g) and female (h) mice. ***p < 0.0001, **p < 0.001, *p < 0.05, *Nr2e1*$^{-/-}$ compared to wild type mice. ###p < 0.0001, ##p < 0.001, #p < 0.05, *Nr2e1*$^{-/-}$ compared to *Nr2e1*$^{+/-}$ mice; &&p < 0.001, *Nr2e1*$^{+/-}$ compared to wild type mice; ANOVA with post hoc Fisher's LSD test. All results are expressed as mean ± SEM. Sample size per sex: wild type (n = 8–14), *Nr2e1*$^{+/-}$ (n = 15–16), *Nr2e1*$^{-/-}$ (n = 6–8).

tion occurs in early life) may impact upon developmental processes [3,8,13] that could thus contribute to the behavioural phenotype. Indeed, early life appears to be a sensitive period to Tlx disruption as indicated by greater neuroanatomical and behavioural impairments in mice with an early life deletion of Tlx compared to mice with an adult knockdown [3,8,11,13]. Thus, the method of interference on Tlx expression may affect the level of impairment observed, and in turn might at least partially explain the inconsistent findings. Taken together, while there is evidence that Tlx plays a role in hippocampus-dependent cognition, adolescence may be the more susceptible period to disruption of spatial working memory and hippocampal processes from Tlx deletion.

During adolescence, both male and female *Nr2e1*$^{-/-}$ mice exhibited impaired cued fear conditioning, a hippocampal-amygdala dependent cognitive process [39]. Interestingly, these deficits persist and are more pronounced in adult male *Nr2e1*$^{-/-}$ mice. On the other hand, the cued fear memory impairments observed in adolescent female *Nr2e1*$^{-/-}$ mice did not persist into adulthood. While *Nr2e1*$^{-/-}$ mice exhibit some delay in cued fear acquisition, freezing behaviour reaches levels exhibited by wild type control mice by the end of the training period (data not shown) and so these impairments are not due to deficits in acquisition. Interestingly, we report the novel finding that male but not female *Nr2e1*$^{+/-}$ mice also exhibited impaired cued fear conditioning during adulthood. Previous studies have reported contradictory findings on cued fear conditioning in male *Nr2e1*$^{-/-}$ mice, with either normal [11] or impaired [7] cued fear conditioning in male mice being reported. Unlike the present study however, the effects in female mice were not investigated in these earlier studies [7,11]. The reasons underlying the discrepancies in adult male *Nr2e1*$^{-/-}$ mice cued fear conditioning are not clear but may again be a function of the methods used to reduce or inhibit Tlx expression or possibly the experimental variables in the cued fear conditioning test. The fear conditioning training protocol employed by Roy et al. consisted of one training session (2 × 30 s; tone 80 dB; 2 kHz; followed by 2 s shock 0.75 mA) whereas the protocol used by Zhang et al. consisted of three training sessions (1 × tone 20 s; 80 dB; 2 kHz; followed by 1 s shock 0.70 mA). In the current study the fear conditioning training consisted of one training session (3 × tone 30 s; 70 dB; 5 kHz; followed by 1 s foot shock 0.65 mA DC current). It is possible that the additional training sessions employed by Zhang et al. facilitated fear association and improved learning compared to the one training session implemented by Roy et al. and in the current study. Furthermore, in the present study the same animals

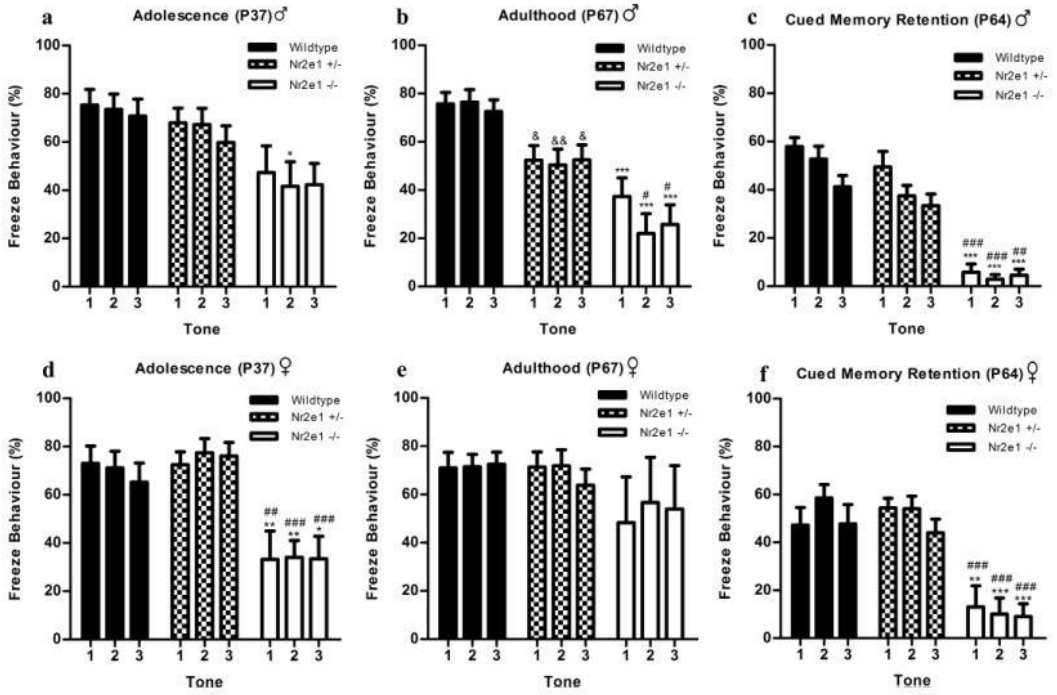

Fig. 6. Cued fear conditioning as a function of genotype during adolescence (P37) and adulthood (P67). Cued fear conditioning in adolescent male (a) and female (d) mice, and in adult male (c) and female (f) mice. Cued fear memory retention test in male (b) and female (e) mice. ***p < 0.0001, ** p < 0.001, * p < 0.05 *Nr2e1*$^{-/-}$ compared to wild type mice. ### p < 0.0001, ## p < 0.001, # p < 0.05 *Nr2e1*$^{-/-}$ compared to *Nr2e1*$^{+/-}$ mice; &p < 0.05 *Nr2e1*$^{+/-}$ compared to wild type miec; ANOVA with post hoc Bonferroni analysis. All results are expressed as mean ± SEM. Sample size per sex: wild type (n = 8–14), *Nr2e1*$^{+/-}$ (n = 13–16), *Nr2e1*$^{-/-}$ (n = 6–8).

were tested during adolescence and in adulthood. It is therefore important to consider the potential effects of fear conditioning training during the adolescent period and its potential impact on fear conditioning in adulthood when drawing conclusions with previous studies. In addition, single housing has been shown to impair contextual and cued fear conditioning [41]. Given that aggression within male Nr2e1$^{-/-}$ mice necessitated being single housed compared to group housed female *Nr2e1* $^{-/-}$ mice, it is not possible to delineate whether the sex-dependent effects on contextual and cued fear conditioning are due to Tlx deletion or housing conditioning *per se*. Nevertheless, since the amygdala plays a key role in cued fear conditions, these studies suggest that Tlx may also be important in regulating the functions of brain structures beyond the hippocampus, particularly during adolescence.

Deletion of Tlx resulted in a sex-dependent effect on thigmotaxis in the open field. Adolescent and adult male but not female *Nr2e1*$^{-/-}$ mice exhibited a significant reduction in thigmotaxis thus suggesting reduced anxiety-like behaviour. In support, previous studies have reported that adult male *Nr2e1*$^{-/-}$ mice with a targeted disruption of the Tlx locus are less anxious within the elevated plus maze [7]. It has also been previously shown that adult male *Nr2e1*$^{-/-}$ mice (with a spontaneous deletion of Tlx) display an anxiolytic phenotype independent of sex, but dependent on strain within the elevated plus maze [8]. Indeed, adult male and female *Nr2e1*$^{-/-}$ mice on a C57BL/6J background exhibited reduced anxiety-like behaviour in the elevated plus maze, while *Nr2e1*$^{-/-}$ mice on a B6129F1 background showed similar exploration to control mice [8]. It is important to note that in the latter study, wild type and heterozygous animals were grouped and constituted the control group, while in the present study differences in cued fear memory were observed in adult male *Nr2e1*$^{+/-}$ mice compared to wild type mice. Thus, when data from *Nr2e1*$^{+/-}$ mice is pooled with that from wild type mice, subtle changes in the limbic system circuitry of *Nr2e1*$^{+/-}$ mice may not be picked up. The amygdala plays a key role in both anxiety behaviour and cued fear conditioning [39,42]. Thus, together with the findings in cued fear conditioning, the reduction in thigmotaxis further supports the hypothesis that Tlx can regulate neurobiological processing in brain areas beyond the hippocampus.

Hyperactivity was observed in both male and female *Nr2e1*$^{-/-}$ mice during adolescence and adulthood. This is in agreement with previous studies using the same strain, where hyperactivity was reported as early as P18 as well as in adulthood [8,13]. The findings presented here, in conjunction with previous reports suggest that in the absence of Tlx, the resulting neuroanatomical disruption causes a sex-independent hyperactivity that occurs in adolescence and persists into adulthood. *Nr2e1*$^{-/-}$ mice exhibited a progressive decline in motor performance on the accelerating rotarod at the onset of adulthood. This novel finding suggests that deletion of Tlx causes disruption of cortico-cerebellar/striatal cognitive processing. However, this disruption does not manifest as behavioural impairment until the onset of adulthood, suggesting that Tlx involvement is age-dependent. The impairment in motor performance on the rotarod does not seem to be

related to the hyperactive phenotype as both adolescent male and female *Nr2e1*$^{-/-}$ mice are hyperactive, yet impairments in rotarod performance only emerge towards the onset of adulthood.

Previous studies using Nr2e1$^{-/-}$ mice with a spontaneous deletion have reported that mice are physically smaller throughout development and adulthood [8,13]. Similarly, we report that both male and female Nr2e1$^{-/-}$ mice exhibit reduced body weight. In addition, we also report that despite smaller body weights, Nr2e1$^{-/-}$ mice exhibit an enhanced growth rate during adolescence. Reduced body weight has also been observed in transgenic mice with a targeted disruption of Tlx, where deviation in postnatal weight gain appears at a similar time point (~P23) to that reported here and previously [6,13]. Specifically, Young et al. have previously reported the body weight of male and female *Nr2e1*$^{-/-}$ mice from embryonic day 12.5 through to adulthood (P70) and show that a deviation in body weight between wildtype and *Nr2e1*$^{-/-}$ mice occurs at approximately P21. However, when a conditional deletion is implemented in adulthood, body weight is not affected [11]. Interestingly, the point of deviation in growth (~P21) coincides with the initiation of hyperactivity (~P18) [13]. Wong et al., suggested failure to gain weight at a similar rate to control littermates may be due to the hyperactive phenotype of these mice as they observed no difference in milk consumption of pre-wean *Nr2e1*$^{-/-}$ mice (P0, P7 and P18). This suggests failure to gain weight at a similar rate was not due to a difference in food consumption [13]. Although in the present study *Nr2e1*$^{-/-}$ mice exhibit a greater growth rate than wild type and *Nr2e1*$^{+/-}$ littermates [13]. It is likely that hyperactivity stems from underlying neuroanatomical abnormalities resulting from germline deletion of Tlx. However, food intake and metabolism studies have yet to be conducted in adulthood which may help delineate the effect of Tlx deletion on body weight gain.

Sensorimotor observations of wild type, *Nr2e1*$^{+/-}$ and *Nr2e1*$^{-/-}$ mice have been previously reported in early postnatal life and adulthood [8]. However sensorimotor performance during the adolescent period had yet to been fully described and any sex-dependent effect had yet to be characterized. Here, we show that both male and female adolescent *Nr2e1*$^{-/-}$ mice exhibit increased provoked biting (an indication of aggression) which is a well-documented phenotype of this strain [8]. However, while biting was increased in both male and female *Nr2e1*$^{-/-}$ mice in adulthood, it did not reach statistical significance (male, $p = 0.14$ and female, $p = 0.13$). Nevertheless, previous studies have reported high aggression in adult male and female *Nr2e1*$^{-/-}$ mice [8,43]. Defective limbic system functionality in *Nr2e1*$^{-/-}$ mice is thought to play a role in the aggressive phenotype [6,8]. We also observed impaired eye reflex in male adolescent *Nr2e1*$^{-/-}$ mice. Mice lacking Tlx display hypoplasia of the retina resulting in impaired vision [5,8]. While previous studies have shown that this impairment is independent of sex, here female *Nr2e1*$^{-/-}$ mice showed a similar response to wild types. It is unclear why this impairment was observed in a sex-dependent manner. Finally, male adult *Nr2e1*$^{-/-}$ mice display impaired piloerection and palpebral closure, while adult female *Nr2e1*$^{-/-}$ mice show similar primary sensorimotor observations compared to wild type and *Nr2e1*$^{+/-}$ littermates. Together, it seems that the sensorimotor impairments (provoked biting, eye reflex, piloerection and palpebral closure) resulting from the Tlx deletion are somewhat dependent on sex and age.

A potential limitation to the behavioural studies within this strain of *Nr2e1*$^{-/-}$ mice is the potential confound of visual impairment [8,44]. Thus, it might be suggested that the anxiolytic phenotype observed within the open field in male *Nr2e1*$^{-/-}$ mice could reflect vision impairments. Specifically, mice with reduced vision may unintentionally explore the centre of the arena because they are unware it is an exposed area of the maze. However, impaired vision has been shown in both sexes [8]. Therefore a lack of a similar anxiolytic phenotype in female mice suggests that this behavioural phenotype is a result of neural abnormalities other than visual abnormalities. Moreover, spontaneous alternation and rotarod performance has previously been shown to be unaffected by visual performance [29,30] and are thus unlikely to be affected by visual impairments in the present study. A second limitation of this study stems from the requirement to single house male knockout mice due to their aggressive phenotype [8]. However, previous studies have shown that spontaneous alternation and motor performance on the rotarod are unaffected by housing conditions in C57BL/6 mice [41]. Furthermore, previous studies have shown that singly housed *Nr2e1*$^{-/-}$ mice exhibit reduced body weight and increased hyperactivity compared to single housed wild type littermates [41] thus suggesting that social isolation does not account for the reduced body weight and hyperactivity of *Nr2e1*$^{-/-}$ mice observed within this study. Notwithstanding that social isolation may impact upon fear conditioning, overall the evidence suggests that the impairments in motor, cognitive and anxiety-related behaviours assessed here are likely a function of Tlx deletion rather than housing conditions.

Given the well-established role of Tlx as a transcriptional repressor of downstream target genes, it is important to consider the molecular mechanisms which may underpin the discrepancies in behaviour between wildtype and Tlx-deficient mice in the current study, and indeed the developmental time points at which these changes emerge. TLX has been shown to recruit the epigenetic modulators lysine-specific histone demethylase 1 (LSD1) and histone deacetylases (HDAC) 3 and 5 to regulate gene expression [45,46]. In turn, expression of an array of genes has been shown to be regulated by Tlx and of particular interest are p21 and Pten as they are involved in adult hippocampal neurogenesis [47,48]. Indeed Pten has been shown to have a role in hippocampal-dependent contextual fear conditioning in mice [49]. Because adolescence is a significant developmental period for the remodelling of hippocampal connectivity and networking including neurogenesis, Tlx-regulated genes such as p21 and pten may have important roles to play in mediating the associated behavioural changes at this time. Future studies will help elucidate this theory.

A number of studies have shown that deletion of Tlx causes neuroanatomical abnormalities similar to those observed in bipolar disorder and schizophrenia, such as enlarged ventricles and reduced volume of the hippocampus, cerebral cortex and amygdala, as well as impaired neurogenesis [50–52]. Moreover, genetic variation at the *Nr2e1* locus in humans has been linked to susceptibility of developing bipolar disorder [26]. Furthermore, the behavioural abnormalities of *Nr2e1* mice are similar to those observed in bipolar disorder i.e., aggression, hyperactivity and impaired learning [53–56]. Interestingly, these disorders manifest primarily during the adolescent period, and this aligns with the behavioural observations in the adolescent Tlx deficient mice in the current study [22]. Thus the observed impairment in limbic system structure and function may indicate a potential role of Tlx in mood disorders. In conclusion, we show that deletion of Tlx results in impairment in motor, cognitive and anxiety-related behaviours during adolescence and adulthood in both male and female mice with the majority of effects occurring during adolescence rather than adulthood. We also show that there is a progressive decline in motor performance of *Nr2e1*$^{-/-}$ mice in adulthood thus indicating cortico-cerebellar/striatal dysfunction in these mice. This novel finding together with alterations in amygdala-dependent behaviour suggests a function for Tlx beyond its regulation of adult neurogenesis in the hippocampus. Adolescence is a critical period for postnatal brain maturation and thus susceptibility to emotional and cognitive-related disorders. Given

that the role of Tlx in the regulation of cognitive and anxiety-related behaviour is most apparent during adolescence, Tlx is poised to be a key target in understanding the emergence of neurobiological disorders at the onset of adolescence and early adulthood.

Conflict of interest

The authors declare no conflict of interest.

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Research report

TLX knockdown in the dorsal dentate gyrus of juvenile rats differentially affects adolescent and adult behaviour

Danka A. Kozareva[a], Tara Foley[a], Gerard M. Moloney[a], John F. Cryan[a,b], Yvonne M. Nolan[a,b,*]

[a] Department of Anatomy and Neuroscience, University College Cork, Ireland
[b] APC Microbiome Ireland, University College Cork, Ireland

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ABSTRACT

The orphan nuclear receptor TLX is predominantly expressed in the central nervous system and is an important factor regulating the maintenance and self-renewal of neural stem cells from embryonic development through adulthood. In adolescence and adulthood, TLX expression is restricted to the neurogenic niches of the brain: the dentate gyrus of the hippocampus and the subventricular zone. The adolescent period is critical for maturation of the hippocampus with heightened levels of neurogenesis observed in rodents. Therefore, we investigated whether lentiviral silencing of TLX expression (TLX knockdown) in the dorsal dentate gyrus of juvenile rats incurred differential impairments in behaviour during late adolescence and adulthood. Our results showed that knockdown of TLX in the dorsal dentate gyrus led to a decrease in cell proliferation in the dorsal but not ventral dentate gyrus.

At a behavioural level we observed differential effects in adolescence and adulthood across a number of parameters. A hyperactive phenotype was present in adolescent but not adult TLX knockdown rats, and an increase in immobility during adolescence and in swimming frequency during adulthood was observed in the forced swim test. There was an increased defecation frequency in the open field during adulthood but not adolescence.

There were no changes in cognitive performance on hippocampus-dependent tasks or in anxiety-related behaviours. In conclusion, silencing of TLX in the dorsal dentate gyrus led to impairments in hippocampal-independent behaviours which either did not persist or were reversed during adulthood. The current data highlight the temporal importance and function of the nuclear receptor TLX during development.

1. Introduction

Adolescence is a critical period during which significant changes in neuroendocrine, neurodevelopmental and behavioural systems take place [1–3]. During this period, neurodevelopmental circuits related to learning and memory, emotional regulation and decision making undergo maturation across the hippocampus [4], amygdala [2] and prefrontal cortex [4,5], thereby rendering changes in cognition related to executive function and cognitive control (For review see [6]). In rodents, adolescence is between postnatal day (P) 21 to P 60; up until P42 is generally considered to be the juvenile period, and P42 to P60 is considered adolescence [6–8]. Within the rodent hippocampus, adolescent development presents with an increase in the number of granule cells and in the volume of hippocampal layers [6,9]. It has been demonstrated in both the human and rodent hippocampus that the number of newborn neurons is up to four times higher during adolescence than the number of neurons born during adulthood [10–12]. This process of neurogenesis (the generation of new neurons from neural stem/progenitor cells (NS/PCs)) occurs throughout the life span of most mammalian species in discrete neurogenic niches of the central nervous system [13]. The subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus is one such neurogenic niche, where newborn granule cells become integrated in the local neuronal network [14]. Ablating and/or decreasing adult hippocampal neurogenesis has been shown to

Abbreviations: CS, conditioned stimulus; DG, dentate gyrus; dDG, dorsal dentate gyrus; EPM, elevated plus maze; E, embryonic day; FST, forced swim test; GCL, granule cell layer; IHC, immunohistochemistry; LVSCR, lentivirus with scrambled shRNA sequence; LVTLXShRNA, lentivirus carrying shRNA to silence TLX; MWM, Morris water maze; NPC, neural precursor cell; NSC, neural stem cell; NDS, normal donkey serum; NGS, normal goat serum; Nr2e1, orphan nuclear receptor subfamily 2 group E member 1; PFA, paraformaldehyde; PBS, phosphate buffered saline; P, postnatal day; SGZ, subgranular zone; US, unconditioned stimulus; VSV-G, vesicular stomatitis virus glycoprotein; vDG, ventral dentate gyrus

* Corresponding author at: Department of Anatomy and Neuroscience, University College Cork, Ireland.
E-mail address: y.nolan@ucc.ie (Y.M. Nolan).

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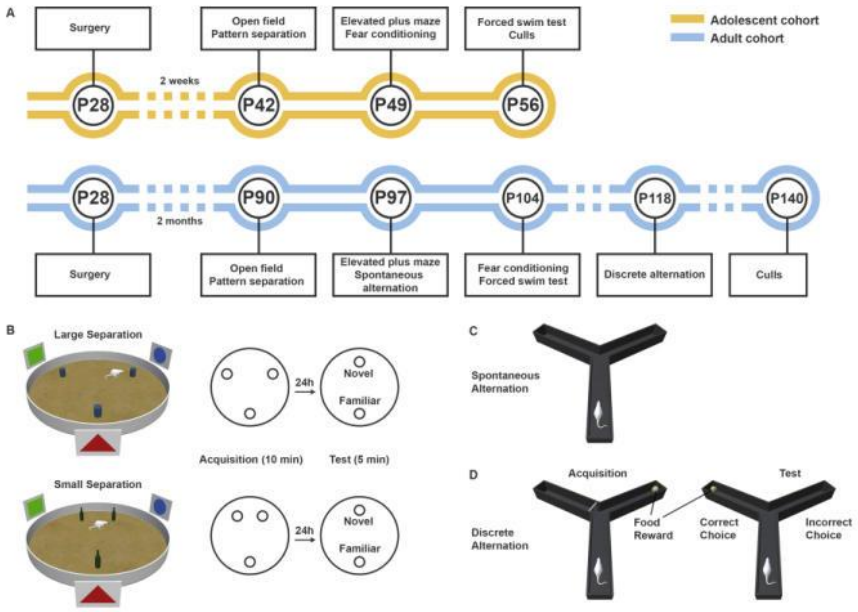

Fig. 1. Experimental design and apparatus.
Outline of the experimental groups and timeline illustrating the duration of the experiment **(A)**. Each circle illustrates the day at which a set of behavioural tests began. The tests were performed over a number of days (depending on the behavioural protocol for each test). Drawing depicting the apparatus used for the pattern separation task including the proximal spatial cues **(B)**. Figure adapted from (Bekinschtein et al., 2014). Drawing depicting the Y-maze apparatus with its three identical arms equidistant at 120° angle from each other **(C)**. Schematic representation of the discrete alternation task in the Y-maze **(D)**.

result in specific deficits such as impairment in long-term memory retention in the Morris Water Maze test in rats (MWM; [15]), decline in performance on contextual fear conditioning tasks in mice [16], impairment in pattern separation in mice [17–19] and increased behavioural despair in the forced swim test in mice (FST; [20]). Ablating hippocampal neurogenesis during adolescence in rats has been shown to result in a decrease in cell proliferation and neuronal survival during adulthood, as well as impaired performance in MWM and fear conditioning tasks [21]. Human studies have also shown that brain irradiation used for cancer treatment during adolescence was associated with long-term decreases in IQ scores and other behavioural changes [22]. Additionally, disorders commonly arising during adolescence such as schizophrenia, drug abuse, attention deficit hyperactivity disorder and depression have been associated with changes in hippocampal neurogenesis (For review see [6]). Currently our understanding of the relative contribution of hippocampal neurogenesis during adolescence to cognitive function and regulation of emotions such as anxiety- and stress-related behaviours and/or disease development during adulthood is limited.

Recent attention has focused on the role of nuclear receptors in neurogenesis. Nuclear receptors are a superfamily of transcription factors that regulate genes involved in physiological and developmental processes and have proven to be important drug targets for a host of diseases [23]. The orphan nuclear receptor subfamily 2 group E member 1 (Nr2e1), commonly known as TLX, is an evolutionary conserved member of the nuclear receptor superfamily found in both vertebrates and invertebrates [24]. An alignment of drosophila, murine and human TLX proteins reveals remarkable interspecies conservation with 70%–99% homology between the three species [25]. Expression of TLX is specific to the developing forebrain and retina. In the mouse embryo, TLX expression is detectable at embryonic day 8 (E8), peaks at E14 and declines thereafter. Postnatal TLX expression increases with high levels present in the neurogenic niches of the adult brain and more specifically in the NPCs in the SGZ and subventricular zone [26,27]. The functional importance of TLX is now apparent from studies showing that TLX maintains adult hippocampal NPCs in a proliferative, undifferentiated state [28,29]. It has also been reported that TLX controls the activation status and the proliferative ability of hippocampal NPCs by repressing cell cycle-related genes such as *pten* [30]. Findings from our lab and others suggest the involvement of TLX in aggression-, cognition- and anxiety-related behaviours as well as in motor performance during adolescence and adulthood [31–34]. We have also shown that deletion of TLX impairs exercise-induced hippocampal neurogenesis during adolescence [35]. Nonetheless, some discrepancies across these studies have emerged due to the time during the lifespan and the location at which the TLX knockdown occurred (spontaneous deletion from birth versus targeted deletion during adulthood, for review see [34]). It has been suggested that an important confounding factor for the spontaneous deletion and homologous recombination deletion models is the fact that TLX signaling has been disrupted from early life when important developmental processes take place. We have also shown that spontaneous deletion of TLX from the embryonic period results in different behavioural outcomes during adolescence and adulthood, respectively, with greater impairments during the adolescent period [33]. Interestingly, distinct hippocampal functions have been associated with the separate anatomical regions of the rodent hippocampus in septo-temporal order [36]. For instance, the dorsal hippocampus has been shown to be primarily involved in spatial learning and memory, while a predominant role of the ventral hippocampus has been displayed in the stress response and anxiety-related

behavior [37,38]. Furthermore, it is now believed that hippocampal neurogenesis may also be segregated along the septo-temporal axis. In support, evidence from rodent studies of behavioural, pharmacological and genetic models of depression suggests that there are regionally associated functional differences in neurogenesis [36,39]. This could potentially account for the discrepancies in findings from TLX deletion studies of neurogenesis-associated behaviours. The aim of the present study was thus to investigate the effect of TLX knockdown using a lentiviral approach to target NPCs in the rat dorsal DG (dDG) during early adolescence on behaviours in late adolescence and adulthood.

2. Materials & methods

2.1. Experimental design

Behavioural analysis was carried out in two cohorts of male rats - adult and adolescent, who had undergone either a targeted silencing of the *Nr2e1* gene (TLX) or GFP-tagged scrambled RNA administration as control in the dDG at P28. Behavioural testing commenced at P42 and P90 for the adolescent and adult cohorts, respectively. Open field test, a modified version of spontaneous location recognition task, elevated plus maze (EPM), cued and contextual fear conditioning and FST were conducted in both cohorts. Only the adult group underwent testing in spontaneous and discrete alternation in the Y maze (see Fig. 1**A** for experimental design).

2.2. Animals

Male Sprague-Dawley rats (n = 10–12) were bred in house, weaned at P21 and group housed on a 12:12-h light-dark cycle in a temperature-controlled colony room (22–24 °C). Food and water were available at all times except when the adult cohort underwent a food restriction protocol for 5 days, during which time they were maintained at 90% of free feeding weight in preparation for the discrete alternation in the Y maze training. All procedures were conducted in accordance with the European Directive 2010/63/EU, and under an authorization issued by the Health Products Regulatory Authority Ireland and approved by the animal Ethics Committee of University College Cork.

2.3. Stereotaxic gene delivery

One week post weaning (P28), rats were anaesthetized with isoflurane/O_2 (2.5–4.5%), and bilaterally stereotaxically infused with 2.0 µl FIV-based short-hairpin RNA vectors (shRNA targeting mouse TLX > 1×10^7 IU mL^{-1} or scrambled control > 1×10^7 IU mL^{-1}) into the dDG (from bregma: AP: -3.3 mm; ML: ± 2 mm; DV: -3.0 mm) at 1 µl/min. Lentiviral particles pseudotyped with the vesicular stomatitis virus (VSV-G) glycoprotein were purchased from Genecopoeia (shRNA Cat# LP-MSH039783-LvU6-0205-m; scrambled control Cat# LP-CSHCTR001-LvU6-0205). The shRNA contained 4 expression constructs designed to silence TLX expression via RNA interference driven by U6 promoter with a guaranteed knockdown effect of 70% or more ($LV^{TLXShRNA}$). The scrambled control contained the same nucleotide sequence, but randomly rearranged, thus non-silencing (LV^{SCR}), and was driven by the same promoter (Genecopoeia, Rockville, MD, USA). Both vectors carried *eGFP* reporter gene clone driven by SV40 and IRES promoters.

2.4. Behavioural tests

2.4.1. Open field test

Spontaneous exploratory locomotor activity and thigmotaxis in the open field were used as a general measure of motor function and anxiety-related behaviours, respectively [40]. At the beginning of each trial, animals were placed in the centre of a circular white open field arena (made in house) having a diameter of 90 cm, for 10 min. The diameter of the centre area was 45 cm. The arena was cleaned with 70% ethanol to avoid cue smell between each trial. The room was brightly lit, which in combination with the exposure to novel environment and open space, induce anxiety in rodents. Thus anxiogenesis was measured by time spent in centre vs. time spent in the edges of the open field arena. Locomotor activity was measured by distance travelled and average speed over the 10minutes of exploration, where a large distance travelled and increased velocity are indicative of hyperactivity [41]. Both thigmotaxis and locomotor activity were measured and analysed using a specialized software with tracking system (Ethovision XT 8.5, Noldus Information Technology, USA).

2.4.2. Pattern separation test

In order to measure the ability of rats to discriminate similar memory representations (a neurogenesis-associated task) [19,80,81] we adopted the modified spontaneous location recognition paradigm, which tests pattern separation [42]. The floor of an open field arena was covered with standard cage bedding, and the light in the room was dimmed. The arena was placed in the middle of the room and was surrounded by three proximal spatial cues and distal standard furniture. Each rat was habituated to the arena and the room for 10 min for 5 consecutive days before the start of the testing. On day 6, the acquisition phase took place, where rats were placed in the arena with 3 identical objects, fixed to the floor of the arena and placed along the circumference 15 cm away from the wall and 30 cm away from the centre of the arena. The objects were either cans or bottles with labels removed, and were cleaned with 50% ethanol between trials. During acquisition, the three objects were either placed equidistant (120°, 49 cm apart) for the large separation task, or two of the objects were placed close together (50°, 20.5 cm apart) and the third object at an equal distance from the other two, for the small separation task. Rats were left to explore the arena with the objects for 10 min and 24 h later the test phase took place. During the test, two objects (identical to the objects used during the acquisition phase) were positioned diametrically opposite to each other. One of the objects was in familiar location, while one was in a novel location. The novel location was chosen such that the object was positioned exactly in between the two familiar locations of the acquisition phase (Fig.1**B**; 180° from the familiar object). Each animal was tested twice in counterbalanced order for the large and small separation. Scores are presented as discrimination ratio, which is calculated by subtracting time spent with familiar object from time spent with novel object divided by total exploration time [42].

2.4.3. Elevated plus maze test

The EPM test is a widely used test of approach-avoidance-related anxiety [40]. The apparatus consists of two open (50.5 × 10.5 cm) and two enclosed (50.5 × 10.5 × 40 cm) arms, which extend from and are connected by a common central square (10.5 × 10.5 cm). The maze was elevated above the floor on a central pedestal (75 cm; Cat# ENV-560; Med Associates Inc). The open arms of the maze are considered to be more aversive for rodents than the closed ones and anxiolytic behaviour is defined as increased number of entries and time spent in the open arms [40]. Animal behaviour was videotaped for the duration of a 5-minute test and the number of entries in closed and open arms was analysed along with the percent time (of total test time) spent in either closed or open arms, respectively. An arm entry was defined as the front two paws of the animal being within the borders of the arm (two-paw criterion).

2.4.4. Y-maze test

The two Y-maze tests described below were performed only on the adult cohort. The experimental apparatus, made in house, was a black Y-maze made of wood. Its arms (50 cm x 10 cm x 30 cm) were connected to form a triangular space in the centre (10 cm x 10 cm x 10 cm), and not belonging to either arm (Fig. 1C). The same apparatus was used

for both tests.

2.4.4.1. Spontaneous alternation in the Y-maze. The spontaneous alternation in the Y-maze test was used to measure spatial working memory and was previously validated in mice [43]. Briefly, each animal underwent one trial and the Y-maze was cleaned with 70% ethanol between animals. The rat was placed into the outward-extending end of one arm (always the same) facing the wall of the arm and was then allowed free exploration of the maze for 5 min. Arms were numbered (1–3) and the sequence of arm entries was recorded manually during the test, where an arm entry was defined by the four-paw criterion [43]. A spontaneous alternation is defined as the consecutive entry in all three arms. The percentage spontaneous alternations, i.e. the ratio of actual to maximum number of alternations completed by the animal within 5 min was calculated [43].

2.4.4.2. Discrete alternation in the Y-maze. The discrete alternation in the Y-maze test is sensitive to dysfunction and/or lesions of the hippocampus as well as to subtle manipulations of hippocampal synaptic plasticity [44]. In the discrete-reward alternation test, successful alternations were rewarded with sugar pellets; the test was performed as documented and validated previously [45]. Briefly, 3 days prior to habituation and testing, rats were placed on a food-restricted diet and reduced to ~90% of their free-feeding weight. During habituation, cage-mate paired rats were placed into the start arm (always the same) of the Y-maze and left to explore freely and collect food rewards. Two food pellets were placed at either end of the two arms that animals were tested in. Rats were then left to explore until they ate the pellets or for 3 min. This was repeated 4 times a day with at least a ten-minute inter-trial interval. During the acquisition day, or forced-trial training [46], each rat was placed in the start arm and forced to visit a baited arm by blocking the access to the other arm (Fig. 1D). The order of left-right forced choices was determined by a pseudorandom sequence. Each trial lasted 1 min or until the rat ate the food pellets and each rat underwent 10 forced trials. During testing, animals received ten trials a day for two consecutive days. The inter-trial interval was 10 min. Each test trial consisted of two runs – a forced run and a free run. The forced run was performed identical to the acquisition day. Ten (test day 1) or sixty (test day 2) seconds after the end of the forced run, the free run was performed. At the beginning of the free run, the rat was returned to the start arm and allowed to choose either goal arm. If the animals chose the arm opposite the one they had been forced into during the forced run, they received food pellets. If the same arm that they had been forced into was chosen, they received no food reward. The percentage correct trials performed by each animal was calculated [45,47].

2.4.5. Fear conditioning: contextual & cued

Hippocampal-dependent learning (contextual) and amygdala-dependent processing (cued) were examined using the fear conditioning paradigm as previously described [48,49]. On day 1 (acquisition), rats were placed into the fear conditioning chambers (Med. Associates, USA: 30.5 cm × 24.1 cm × 21 cm) which were fragranced with lemon and ginger from a tea bag placed under the grids of the flooring. Animals were allowed to explore the chamber for two minutes during the habituation period, followed by the delivery of three foot shocks paired with tones (30 s tone: 5 kHz; 70 dB; 1 s foot shock: 0.60 mA DC current) separated by 30-second intervals. One minute after the last shock, animals were removed from the chambers and placed back in their cages. On day 2, contextual fear memory was measured by placing the rats back in the same chambers for 5 min and 30 s, but in the absence of tone-shock pairing. On day 3, cued fear conditioning was assessed by placing the animals in the same chambers but changing the context to a novel one (white solid floor; black wall insert at 60°; almond scent). Two minutes after the start of the test, three tones were presented without being paired with shock (30 s: 5 kHz; 70 dB). Behaviour was recorded and the percentage time spent in freeze position was calculated with specialized software (Video freeze, Med Associates, USA) for the duration of the whole test or during the 30-second tone presentations for the context and cued fear conditioning test, respectively.

2.4.6. Forced swim test

The FST was performed as previously described [50]. In brief, rats were placed individually in Pyrex cylinders (Fischer Scientific: 21 cm × 46 cm) for 15 min. Cylinders contained water at 25 °C up to a 30 cm mark on the cylinder. Behaviours were recorded by video camera for subsequent analysis. The 15-min session was divided into three separate triads and was analysed as previously described [51]. For this purpose, a time-sampling technique was employed, whereby the predominant behaviour (immobile, swimming, or climbing) in each 5-second period of the 5-minute interval was recorded. When the rat performed minimal movements (or none at all) to keep its head afloat, the behaviour was scored as immobile. When the rat was moving horizontally through the chamber crossing quadrants, swimming behaviour was assigned. Finally, climbing was defined as upward-directed movements of the forepaws along the side of the swim chamber. Scores are presented as cumulative for each triad and for the full 15-minute duration of the test.

2.5. Tissue preparation

At P56, rats from the adolescent cohort were euthanized with an intraperitoneal injection of anaesthetic (Pentobarbital: 50 mg/kg) and transcardially perfused using a 0.1 M phosphate buffered saline (PBS) solution followed by 4.0% paraformaldehyde (PFA) in PBS. After overnight incubation in PFA, brains were incubated in 30% sucrose until they sank, and subsequently flash frozen using liquid nitrogen. At P140 rats from the adult cohort were sacrificed by rapid decapitation (n = 8 of each group). Freshly dissected hippocampal tissue from the adult rats was processed for molecular analysis by qRT-PCR as described below. In order to validate virus expression was regionally sustained into adulthood, n = 2 of each group were perfused and the brains were processed as described above.

2.6. Immunohistochemistry

Coronal sections (40 µm) through the hippocampus from all perfused brains were collected onto slides in a 1:12 series, then stored at −80 °C. To confirm virus spread and silencing of TLX, sections were assessed for GFP and TLX immunoreactivity, respectively. Sections were washed, incubated in 3% normal donkey serum (NDS: Sigma D9663) and then in anti-GFP raised in rabbit (GeneTex; GTX113617; 1:250) or anti-TLX raised in rabbit (Abcam ab86276; 1:100) antibodies overnight at 4 °C. Following that, sections were washed and incubated in AlexaFluor488 donkey anti-rabbit (ab150073; 1:500) antibody for two hours at room temperature. Subsequently, sections were washed, counterstained with DAPI (Sigma D9642; 1:5000) and coverslipped using anti-fade mounting medium (Dako; S3023). To assess cell proliferation, immunohistochemistry (IHC) for the Ki-67 antigen was performed. Briefly, sections were incubated in H_2O_2 (in 1% methanol, 40 min at room temperature) and then blocked in 10% normal goat serum (NGS; Sigma; G9023). Sections were incubated with rabbit anti-Ki67 antibody (GeneTex; GTX16667; 1:250; overnight at 4 °C), followed by sequential incubations with the streptavidin-biotin immunoenzymatic antigen detection system (Abcam; ab64261) and coverslipped using DPX mounting medium (Sigma; 44,581).

2.7. Image analysis and cell quantification

Fluorescent images were obtained with Olympus FV1000 scanning laser confocal system (BioSciences Imaging Centre, Department of Anatomy and Neuroscience, University College Cork, Ireland),

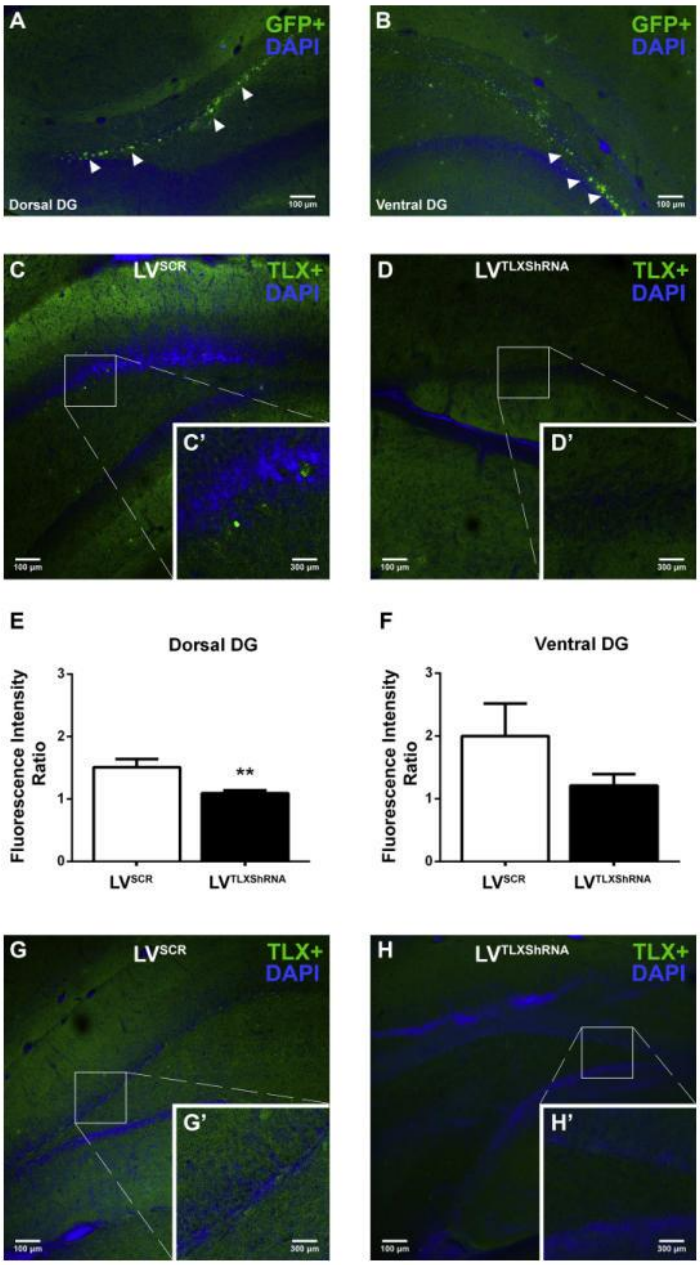

Fig. 2. Lentiviral transduction of the rat dDG resulted in TLX knockdown.
Representative confocal images illustrating virus localization after stereotaxic surgery. Immunohistochemical staining shows GFP + (green; arrowheads) cells and DAPI + (blue) nuclei in the dorsal **(A)** and ventral **(B)** DG of $LV^{TLXShRNA}$ treated rats. Images were taken at 10X magnification. Scale bar = 100 µm. Representative confocal images of coronal sections through the dDG immunohistochemically stained with TLX (green) and DAPI (blue) from adolescent control (LV^{SCR}) **(C)** and $LV^{TLXShRNA}$ **(D)** rats. TLX fluorescence intensity measured as ratio of fluorescence intensity within the DG divided by fluorescence intensity outside of the DG in the dorsal **(E)** and ventral **(F)** DG of adolescent rats transduced with either LV^{SCR} (white bars) or $LV^{TLXShRNA}$ (black bars). Data are expressed as mean ± SEM. ** $p < 0.01$; independent-sample *t*-test, n = 6–8 for dDG and n = 3–7 for vDG. Representative confocal images of coronal sections through the dDG immunohistochemically stained with TLX (green) and DAPI (blue) from adult LV^{SCR} **(G)** and $LV^{TLXShRNA}$ **(H)** rats. Images were taken at 10X magnification. Scale bar = 100 µm. Insets **(C', D', G', H')** depict zoomed in image of areas selected (white square) from the respective lower magnification images (C, D, G, H, respectively). Scale bar = 300 µm.

incorporating an IX81 inverted microscope. Z-stack images with 4.4 µm step size were collected using a 10X UPlanSApo objective, which has a NA of 0.40 and is highly corrected for both spherical and colour aberration. The excitation light source used was a laser diode for 405 nm and a multiline argon laser for 488 nm. The filter set used was an excitation dichroic mirror set DM 405/488/543. Emission light was detected using a carriable barrier filter, which for DAPI and Alexa Fluor 488 was set to 425–475 nm and 500–530 nm, respectively. Emission

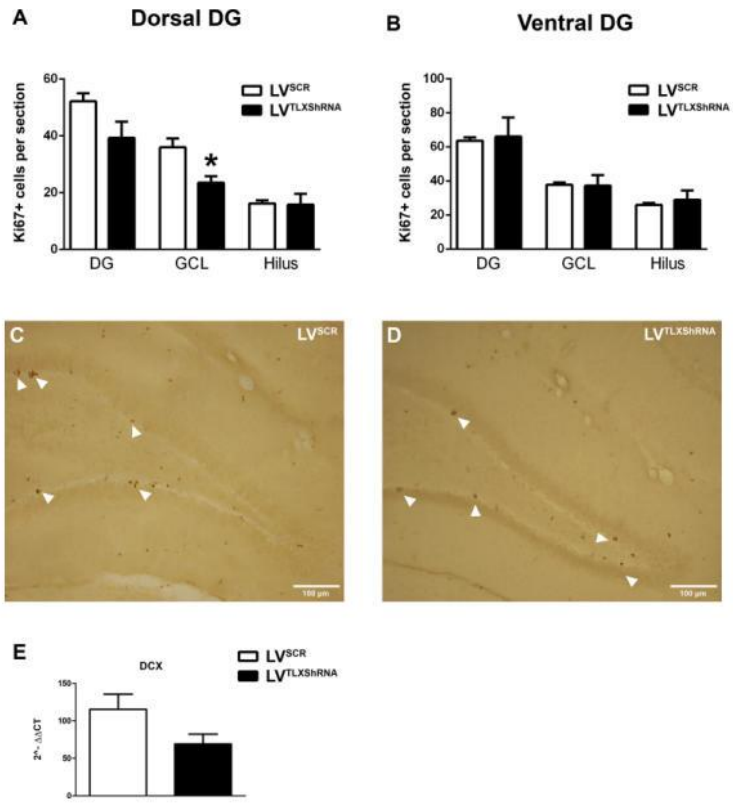

Fig. 3. Lentiviral transduction of the rat dDG reduced number of proliferating cells in the dDG.
Mean number of Ki67 + cells per section in the dorsal **(A)** and ventral **(B)** DG of adolescent rats transduced with either LV^{SCR} (white bars) or $LV^{TLXShRNA}$ (black bars). Data are expressed as mean ± SEM. * $p < 0.05$; independent-sample *t*-test, n = 4. Representative bright field images of coronal sections through the DG immunocytochemically stained with Ki67 (dark brown; arrowheads) from LV^{SCR} treated **(C)** and $LV^{TLXShRNA}$ treated adolescent rats **(D)**. Image taken at 10X magnification. Scale bar = 100 µm. Relative mRNA expression of DCX in the hippocampus of $LV^{TLXShRNA}$ and LV^{SCR} adult rats. All values were adjusted to relative expression of the housekeeping gene *Actb* (*E*).

light was detected with three photomultiplier detectors. The software used for image acquisition was Olympus Fluoview Ver4.2b. For bright field images, an Olympus BX533 upright microscope coupled to an Olympus DP72 camera was used with a 10X objective. The DG was imaged bilaterally on all sections.

In order to quantify TLX staining through the whole DG, mean fluorescence intensity was measured across a randomly selected area within the DG and the mean background fluorescence intensity was measured across a randomly selected area of the same size outside the DG. TLX staining was expressed as the ratio between fluorescence within to fluorescence outside of the DG. For cell quantification, systematic random sampling was used through the whole DG by counting the cells on both hemispheres in 1:12 series (480 µm apart). Analysis of both fluorescence intensity and cell numbers was performed using the image software package ImageJ (National Institute of Health, USA). Cell numbers and fluorescence intensity ratio were expressed as an average per section in both the dDG and vDG according to the coordinates: dDG as -2.8 to – 4.0 from Bregma; vDG as −4.0 to −6.3 [52].

2.8. Quantitative RT-PCR analysis of hippocampal tissue

Samples were processed according to the GenElute kit protocol (Sigma; RTN350). Briefly, total cellular RNA was homogenized into lysis solution and the homogenized sample was filtered through a binding column to remove non-RNA from the sample. 70% ethanol was added to the filtrate and purified through columns, which were then washed with buffer. Purified mRNA was recovered into 30 µL of elution solution. Samples were further treated with DNase1 (Sigma; AMPD1) to ensure the complete removal of endogenous DNA from the samples. Total RNA yield and purity were determined using the Nanodrop System (Thermo Scientific). Synthesis of cDNA was performed using 0.5 µg of normalized total RNA from each sample using ReadyScriipt cDNA synthesis mix (Sigma; RDRT-25RXN). Probed cDNA amplification was performed in a 20 µL reaction consisting of 10 µL KiCqStart qPCR Mix with ROX (Sigma; KCQS02), 0.1 µL of each forward and reverse primer (final concentration 250 nM), 1 µL cDNA template, and 8.8 µL RNase-free water. Real time RT PCR was performed in duplicate in a 96-well plate (Applied Biosystems) and captured in real time using the StepOne Plus System (Applied Biosystems). Relative gene expression was adjusted to the housekeeper *Actb* (Actin Beta), and quantified using the $2^{-\Delta\Delta CT}$ method [83].

2.9. Statistical analysis

All data were analysed using SPSS statistical software (SPSS 17.0, Chicago, IL). Data were analysed by an independent-sample *t*-test except in the case of cued fear conditioning, when repeated measures ANOVA was employed followed by Bonferoni post hoc analysis. Group means from pattern separation test were compared against chance level by one-sample *t*-test. An alpha level of 0.05 was used as criterion for statistical significance. All data are presented as mean ± SEM.

3. Results

3.1. Lentiviral transduction of the rat dDG resulted in TLX knockdown and a reduced number of proliferating cells in the dorsal granule cell layer

GFP was expressed in both the dorsal and ventral hippocampus of all adolescent rats (n = 10–12). Representative images show that GFP + cells were evident in the SGZ of both the dDG (Fig. 2A) and vDG (Fig. 2B) of all animals at four weeks following surgery, demonstrating successful transduction of cells in the neurogenic niche by the lentivirus (Fig. 2**A-B**, white arrowheads). Representative images show reduced fluorescence intensity of TLX after probing with TLX antibody by IHC in the DG of rats that underwent TLX knockdown ($LV^{TLXShRNA}$ (Fig. 2D)) compared to control (LV^{SCR} treated) rats (Fig. 2C). TLX expression was significantly decreased in the dDG (t (12) = 3.456, $p < 0.05$; Fig. 2E) but not vDG (Fig. 2F) in $LV^{TLXShRNA}$ compared to control (LV^{SCR})-treated rats. Representative images of TLX immunohistochemistry in the adult hippocampus show a decrease in TLX immunostaining in $LV^{TLXShRNA}$ (Fig. 2H) compared to control rats (Fig. 2G) demonstrating that TLX knockdown was sustained into adulthood.

To validate that TLX knockdown induced an impairment in cell proliferation, Ki67-immunopositive cells were counted in adolescent rats that had been injected with either $LV^{TLXShRNA}$ or LV^{SCR}. $LV^{TLXShRNA}$ rats had on average 39.28 proliferating cells (SD = 11.37) in the whole of the dDG, while the mean number of Ki67 + cells in the dDG of control rats was 52.17 (SD = 5.7). Despite this difference not being significant, the effect size was moderate to strong (r = 0.4; Fig. 3A). The number of proliferating cells in the whole vDG was not affected by $LV^{TLXShRNA}$ treatment during adolescence (Fig. 3B). There was a significant decrease in Ki67 + cells in the dorsal granular cell layer (GCL (t (6) = 3.203, $p < 0.05$; Fig. 3A) but not the ventral GCL (Fig. 3B) of $LV^{TLXShRNA}$ treated rats compared to the respective dorsal and ventral GCLs of the control group. No differences in the mean numbers of Ki67 + cells were observed in the hilus across the septo-temporal axis (Fig. 3**A-B**). The representative image of the DG of control rats shows multiple clusters of Ki67 + cells (Fig. 3**C**; arrowheads), while fewer numbers of cells can be observed in the DG of $LV^{TLXShRNA}$ rats (Fig. 3**D**; arrowheads). Furthermore, a trend towards a decrease in DCX expression measured by qRT-PCR was observed in the hippocampi of TLX knockdown compared to control rats (Fig. 3E).

3.2. TLX knockdown during adolescence increased locomotor activity in adolescent but not adult rats

Adolescent rats that had been treated with TLX knockdown lentivirus ($LV^{TLXShRNA}$) travelled a significantly greater distance (cm) in the open field arena than control (LV^{SCR} treated) rats ($M_{LV}^{TLXShRNA}$ = 7061, $SD_{LV}^{TLXShRNA}$ = 1490; M_{LV}^{SCR} = 5043, SD_{LV}^{SCR} = 779; t (20) = 3.854, $p < 0.001$; Fig. 4A). $LV^{TLXShRNA}$ treated adolescent rats also moved at a significantly greater velocity than the control rats ($M_{LV}^{TLXShRNA}$ = 12.01, $SD_{LV}^{TLXShRNA}$ = 2.54; M_{LV}^{SCR} = 9.27, SD_{LV}^{SCR} = 1.19; t (20) = 3.117, $p < 0.01$; Fig. 4C). During adulthood, there was a trend towards a decrease in the distance travelled in $LV^{TLXShRNA}$ rats compared to controls ($M_{LV}^{TLXShRNA}$ = 4041, $SD_{LV}^{TLXShRNA}$ = 1139; M_{LV}^{SCR} = 4851, SD_{LV}^{SCR} = 838; Fig. 4B) and there was no difference in velocity between the two groups (Fig. 4D).

3.3. TLX knockdown during adolescence did not affect anxiety-related behaviours in adolescent rats but increased defecation in the open field in adult rats

Thigmotaxic behaviour remained the same across treatments and age; both adolescent and adult rats spent similar time in the centre of the open field regardless of TLX knockdown (Fig. 5**A-B**). Anxiety during the open field test was also measured by counting the number of faecal pellets in the arena after each animal had been exposed to the test. TLX knockdown did not affect the number of faecal pellets in adolescent rats (Fig. 5C), but induced a significant increase in the number of pellets compared to the control (LV^{SCR} treated) group when assessed in adulthood (t (18) = 2.010, p = 0.05; Fig. 5D).

Anxiety-like behaviour in the EPM did not differ between adolescent $LV^{TLXShRNA}$ or LV^{SCR} treated rats. Specifically, $LV^{TLXShRNA}$ treated rats spent the same amount of time (percent of total) in the anxiolytic open arms of the maze as their control counterparts (Fig. 5E). Furthermore, the number of entries made to the open arms was the same across both treatment groups (Fig. 5G) as was the total number of entries made ($M_{LV}^{TLXShRNA}$ = 27.75, $SD_{LV}^{TLXShRNA}$ = 8.65; M_{LV}^{SCR} = 24.11, SD_{LV}^{SCR} = 5.79). During adulthood, TLX knockdown did not affect the time rats spent in the open arm ($M_{LV}^{TLXShRNA}$ = 34.08, $SD_{LV}^{TLXShRNA}$ = 13.68; M_{LV}^{SCR} = 41.94, SD_{LV}^{SCR} = 9.37; Fig. 5F). Adult $LV^{TLXShRNA}$ rats showed trends towards reduced number of entries to the open arms ($M_{LV}^{TLXShRNA}$ = 14.4, $SD_{LV}^{TLXShRNA}$ = 4.71; M_{LV}^{SCR} = 17.8, SD_{LV}^{SCR} = 3.15; Fig. 5H) and reduced total number of entries ($M_{LV}^{TLXShRNA}$ = 29.7, $SD_{LV}^{TLXShRNA}$ = 7.71; M_{LV}^{SCR} = 35.3, SD_{LV}^{SCR} = 5.73).

3.4. TLX knockdown increased immobility and reduced swimming frequency in adolescent rats, and increased swimming frequency in adult rats

There was no significant difference in performance between $LV^{TLXShRNA}$ and control (LV^{SCR} rats at adolescence or adulthood in the first triad of the forced swim test. Thus, during the first 5 min of the test, all animals displayed comparable mean scores on immobility frequency, swimming frequency, and climbing frequency (Fig. 6). During the second triad, immobility frequency remained the same across treatments for both age cohorts (adolescent: Fig. 6**A**; adult: Fig. 6B). Similarly, climbing frequency did not differ across groups (adolescent: Fig. 6**E**; adult: Fig. 6F). There was no difference in swimming frequency between groups at adolescence (Fig. 6C), or adulthood (Fig. 6D). However, in the last 5 min of the test, adolescent $LV^{TLXShRNA}$ rats exhibited a significantly lower score on swimming frequency behaviour (t (20) = 2.726, $p < 0.05$, Fig. 6C) which was coupled with a significant increase in immobility frequency (t (20) = 2.381, $p < 0.05$, Fig. 6A) compared to the control rats. Adult $LV^{TLXShRNA}$ rats on the other hand, did not differ in their immobility score (Fig. 6B) but they did display a change in swimming frequency (t (18) = 2.242, $p < 0.05$) compared to controls (Fig. 6D). There was no change in the frequency of climbing behaviour across groups in this last phase of the test (adolescent: Fig. 6**E**; adult: Fig. 6F).

3.5. TLX knockdown during adolescence did not affect performance in the pattern separation task or the contextual and cued fear conditioning paradigm in adolescent or adult rats

$LV^{TLXShRNA}$ treatment had no effect on performance in the pattern separation task by adolescent rats in either the large separation or the neurogenesis-associated small separation (Fig. 7A). Pattern separation refers to the ability to distinguish and store similar inputs as distinct representations in memory [53]. One-sample t-test revealed that neither of the groups performed significantly different from chance level in both versions of the test. No difference in performance on the large separation task was observed between adult $LV^{TLXShRNA}$ or control ($LV^{SCR)}$ rats; Fig. 7B). In addition, neither group could discriminate between the novel and familiar locations as determined by one-sample t-test comparing the average of each group to chance performance. There was no change in the performance of adult rats treated with $LV^{TLXShRNA}$ compared to LV^{SCR} in the small separation task (Fig. 7B). The performance of both groups was at chance level as determined by an one-sample t-test.

No changes in the freezing behaviour of $LV^{TLXShRNA}$ treated rats were observed during the context test of the fear conditioning

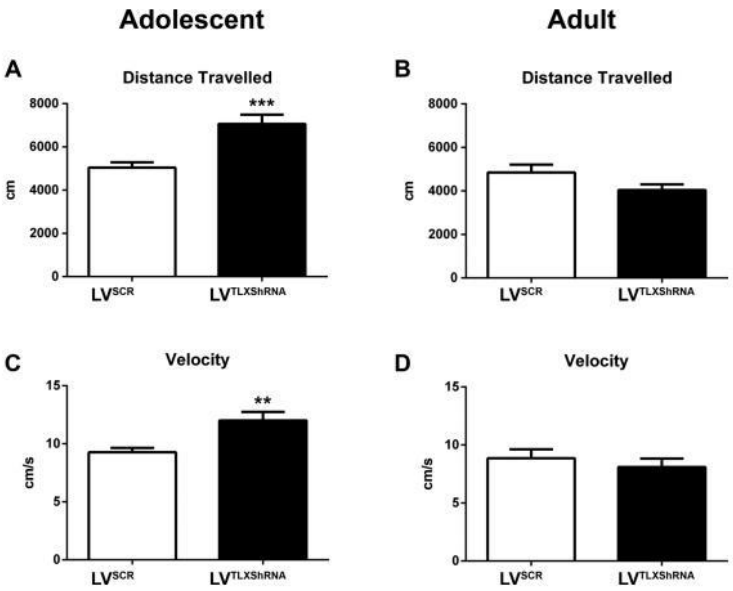

Fig. 4. TLX knockdown during adolescence increased locomotor activity in adolescent but not adult rats.
Distance travelled in the open field arena (cm) during adolescence **(A)** and adulthood **(B)** in LV^{SCR} treated (white bars) and $LV^{TLXShRNA}$ treated (black bars) rats. Velocity during exploration of the open field arena (cm/s) during adolescence **(C)** and adulthood **(D)** in LV^{SCR} treated (white bars) and $LV^{TLXShRNA}$ treated (black bars) rats. *** $p < 0.001$, ** $p < 0.01$; independent-sample *t*-test. All results are expressed as mean ± SEM. Sample size per age: adolescent (n = 10–12) and adult (n = 10).

paradigm. Both adolescent and adult $LV^{TLXShRNA}$ rats were motionless for a similar proportion of time as the control rats (Fig. 7D). The Mauchly's test indicated that the assumption of sphericity had been violated for the cued fear conditioning data for the adolescent cohort (χ^2 (2) = 15.024, $p < 0.01$). Therefore, the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity as the epsilon was less than 0.75 ($\varepsilon = 0.707$). The results showed that rats in both treatment groups maintained a high percent freezing time throughout the presentation of all three tones (Fig. 7E). Furthermore, rats treated with $LV^{TLXShRNA}$ exhibited similar cued fear recall response as the control rats (Fig. 7E). There was no within-subject effect of the repeated tone presentations on freezing behaviour in the adult cohort (Fig. 7F). Lastly, both groups retained the same level of cued fear recall (Fig. 7F).

3.6. TLX knockdown during adulthood did not impair behaviour in the Y-maze

Spontaneous alternation behaviour, a measure of spatial working memory, was tested in adult rats treated with $LV^{TLXShRNA}$. TLX knockdown showed no significant effect on the alternation rates when compared to the control rats (Fig. 8A). Furthermore, both groups exhibited a comparable number of arm entries (Fig. 8B) indicating similar motivation states and total activity in all animals. Since the alternation rates for both $LV^{TLXShRNA}$ and control rats did not reach the criteria of 75% [45], the modified discrete or rewarded alternation test of working memory was performed in the Y-maze. TLX knockdown had no influence on the percent of correct choices made in the discrete alternation immediately after (Fig. 8C) or one minute after (Fig. 8D) an animal had undergone the sample/forced-run phase.

4. Discussion

The results from these experiments show that lentiviral transduction successfully diminished TLX protein expression in the rat DG, which was associated with a decrease in the number of proliferating cells in the dGCL. We show that TLX knockdown during adolescence induced hyperactivity in adolescent but not adult rats. With respect to anxiety-related behaviours measured by EPM and thigmotaxis in the open field, we found no differences between groups across the two age cohorts. However, there was a significant increase in defecation in the open field arena by $LV^{TLXShRNA}$ treated adult rats. The active behaviours in the forced swim test did not differ until the final triad or the last 5 min of the 15-minute test. During this time frame, we observed an increased frequency in immobility behaviour and decreased frequency in swimming behaviour by $LV^{TLXShRNA}$ treated rats compared to the LV^{SCR} treated controls during late adolescence. On the contrary, $LV^{TLXShRNA}$ treated animals exhibited an increase in swimming frequency in adulthood. TLX knockdown did not hinder performance on the pattern separation task or the contextual and cued fear conditioning paradigm, neither during adolescence, nor adulthood. Additionally, performance on the spontaneous and discrete alternation in the Y-maze task during adulthood was also not affected by silencing of the TLX gene. Thus, these data confirm that targeted disruption of the TLX gene within the dDG during early adolescence can have differential effects on behaviour during late adolescence and adulthood, without affecting learning and memory at either age. The current data thus highlight the temporal nature and function of the nuclear receptor TLX during development.

Lentiviral vectors are an appealing vehicle for shRNA delivery whereby stable knockdown of a gene has been achieved with high efficiency in many different mammalian cell types [54,55]. Here we delivered a lentiviral shRNA vector designed for mouse TLX gene into the rat hippocampus. The gene homology for TLX between species is very high with 100% identity in the DNA-binding domain and 98% identity in the ligand-binding domain between mouse and rat [25]. The high homology across species has also been demonstrated in a study where the aggressive phenotype of mice with a spontaneous deletion of the gene was rescued by expression of its human homologue [56]. The efficiency of the knockdown was confirmed by a decrease in fluorescence intensity of immunohistochemically labelled TLX protein in the adolescent cohort. TLX is a major regulator of NSC proliferation [29]. We confirmed this role of TLX in the hippocampus by examining the

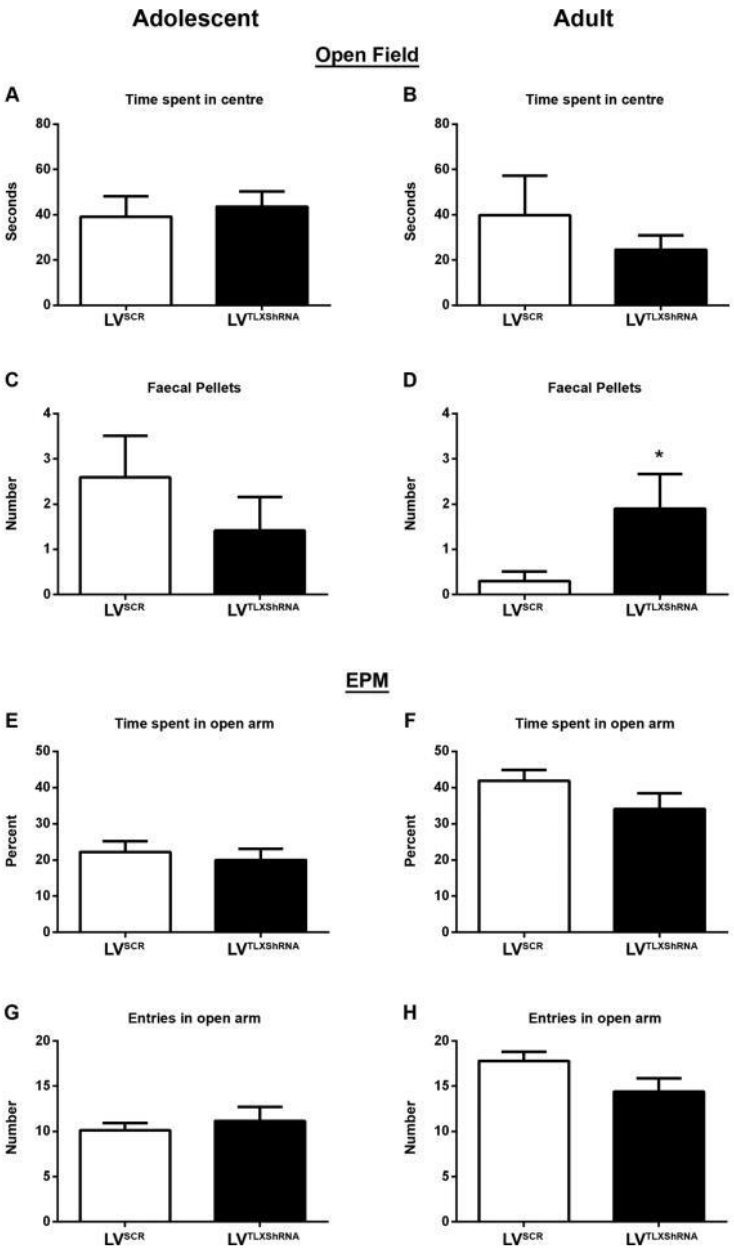

Fig. 5. TLX knockdown during adolescence did not affect anxiety-related behaviours in adolescent rats but increased defecation in the open field in adult rats.
Thigmotaxis and number of faecal pellets in the open field as measures of anxiolytic behaviour during adolescence (time spent in centre: **A**, number of pellets: **C**) and adulthood (time spent in centre: **B**, number of pellets: **D**). Percent time spent in the open arms and number of entries to the open arms during the EPM task as measure of anxiety-like behaviour during adolescence (% time in open arm: **E**; number of entries to open arm: **G**) and during adulthood (% time in open arm: **F**; number of entries to open arm: **H**). White bars – LV^{SCR} treated rats, black bars – $LV^{TLXShRNA}$ treated rats. * $p < 0.05$; independent-sample *t*-test. All results are expressed as mean ± SEM. Sample size per age: adolescent (n = 10–12) and adult (n = 10).

density of Ki67 + cells in $LV^{TLXShRNA}$ treated rats and showed that there was a significant reduction in the number of proliferating cells in the dDG but not the vDG. It is important to note that neurogenesis is a multifactorial process of cell proliferation, differentiation and neuronal integration. Thus it may be possible that knocking down one of these factors alone (as in this case TLX knockdown targets proliferating NPCs) is not sufficient to enhance hippocampal-associated behaviour or the other stages of neurogenesis such as differentiation and neuronal integration. Moreover, inhibiting one specific component of neurogenesis may not translate into a decrease in differentiation and/or integration

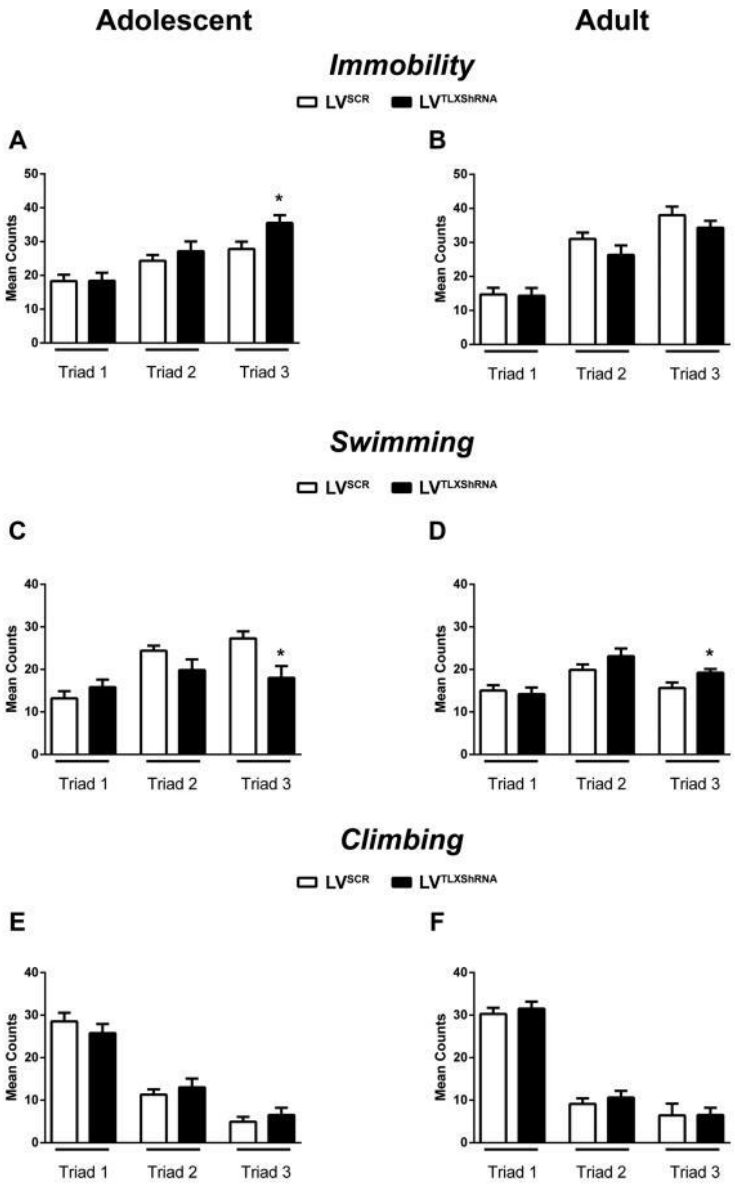

Fig. 6. TLX knockdown increased immobility and reduced swimming frequency in adolescent rats, and increased swimming frequency in adult rats in the forced swim test.
Active behaviours in the forced swim test for Triad 1 (0–5 min), Triad 2 (5–10 min) and Triad 3 (19-15 min). Immobility behaviour frequency for adolescent **(A)** and adult **(B)** rats, swimming behaviour frequency for adolescent **(C)** and adult **(D)** rats, and climbing behaviour frequency for adolescent **(E)** and adult **(F)** rats treated with LV^{SCR} (white bars) or $LV^{TLXShRNA}$ (black bars). * $p < 0.05$; independent-sample *t*-test. All results are expressed as mean ± SEM. Sample size per age: adolescent (n = 10–12) and adult (n = 10).

of the newborn cells. For instance, it has been shown that overexpressing the transcription factor Sonic Hedgehog (Shh) in NPCs, resulted in increased cell proliferation but no change in the number of differentiating neurons [84]. Similarly, we only observed a trend towards, rather than a significant decrease in the expression of hippocampal DCX in our adult cohort. It has been demonstrated that TLX prevents NPC differentiation by sustaining them in a proliferative state [27,28,30]. Our behavioural and immunohistochemical data show that despite the decreased cell proliferation brought about by TLX knockdown, there was no decrease in neurogenesis overall. Rather, it appears that inhibition of the proliferating NPCs was achieved without significantly altering the differentiation status or the number of newborn neurons. This is in line with the work on Shh by Lai et al. [4]. It could also explain why we did not observe any effect of TLX shRNA in the pattern separation task, which relies on functional newborn granule cells. It further reinforces the concept that for any impairment in

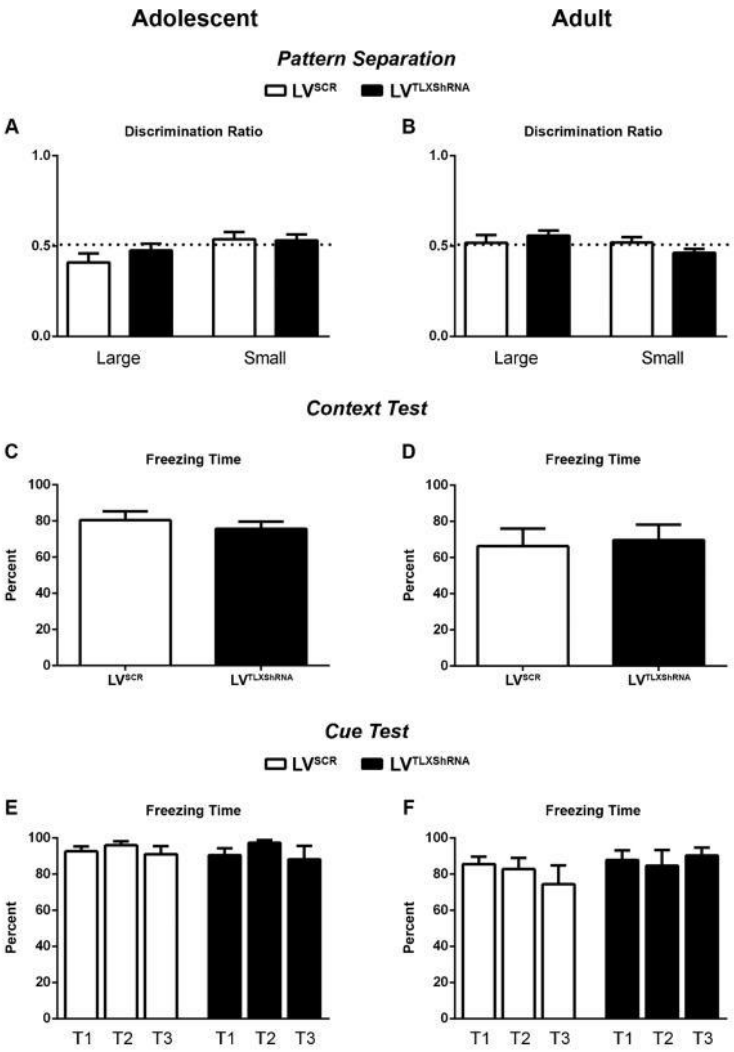

Fig. 7. TLX knockdown did not affect performance in pattern separation and contextual and cued fear conditioning during adulthood and adolescence.
Performance on the large and small pattern separation task during adolescence **(A)** and adulthood **(B)**. Contextual freeze behaviour in the fear conditioning paradigm during adolescence **(C)** and adulthood **(D)**. Cued fear conditioning during adolescence **(E)** and adulthood **(F)**. All results are expressed as mean ± SEM and were analyzed with an independent-sample *t*-test, except in **(E)** and **(F)** when repeated measures ANOVA was employed followed by Bonferoni post hoc analysis. Data in **(A)** and **(B)** were also compared against chance level (dashed line) by one-sample *t*-test. Sample size per age: adolescent (n = 10–12) and adult (n = 10). White bars – LV^{SCR} treated rats, black bars – $LV^{TLXShRNA}$ treated rats.

hippocampal cognitive function to be induced by altering NPCs, there must be a robust inhibition of all stages of neurogenesis.

Silencing TLX expression in the juvenile rat dDG was associated with a hyperactive phenotype during late adolescence but not adulthood. We and others have previously shown that mice with spontaneous deletion of the TLX gene display hyperactivity during adolescence and adulthood [33,57,58]. When TLX was silenced in the hippocampus of two-month-old mice by inducible recombination, and locomotor behaviour was examined later in adulthood, no hyperactivity or motor impairments were observed [32]. This corroborates our findings from the adult rat cohort. Thus, in the absence of TLX from the embryonic period, hyperactivity occurs throughout life. When TLX is silenced during the juvenile period hyperactivity only manifests for short period after the knockdown of the gene, and when TLX is silenced in adulthood the hyperactive phenotype is not present. Interestingly, in another mouse model where TLX was knocked out prior to E12 through homologous recombination, hyperactivity was not observed despite the presence of impairments in forebrain development and deficiencies in emotional behaviour [59]. Remarkably, the impairments observed in the homologous recombination model matched those observed in the spontaneous deletion model except that an intact striatum was evident in the former but not the latter mouse model [33,34,57,59,60]. The striatum plays a crucial role in the differential processing of reward-seeking and risk-taking behaviour during adolescence and adulthood [61,62], which may be modulated by differential pruning of dopamine receptors [63]. Due to the connectivity between the hippocampus and striatum [64,65], it is possible that the targeted silencing of TLX in the dDG of the juvenile rats in the present study caused changes to striatal

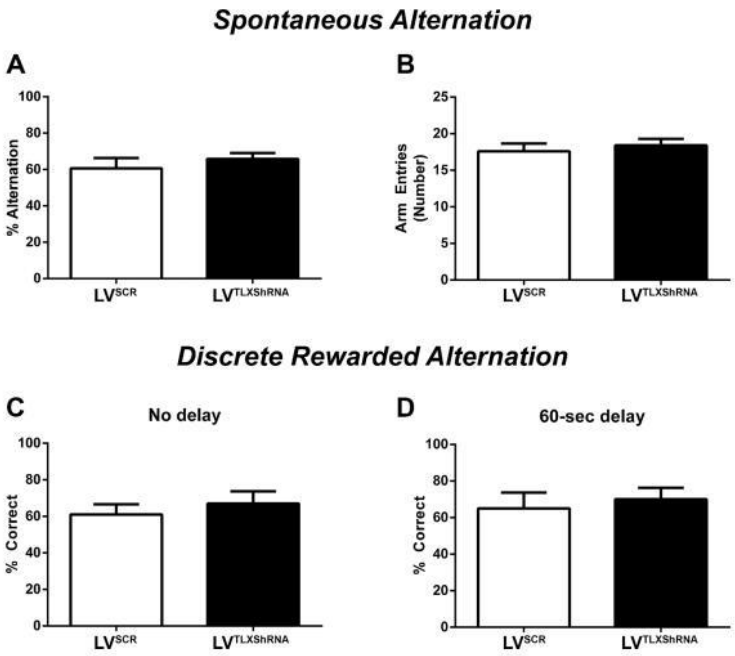

Fig. 8. TLX knockdown during adulthood did not affect behaviour in the Y-maze.
Percent spontaneous alternation in the Y-maze **(A)** during adulthood and number of arm entries **(B)** for LV^{SCR} treated (white bars) and $LV^{TLXShRNA}$ treated (black bars) adult rats. Percent correct choices made during the discrete rewarded alternation task in the Y-maze without **(C)** or with **(D)** 60-second delay in LV^{SCR} treated (white bars) and $LV^{TLXShRNA}$ treated (black bars) adult rats. All results are expressed as mean ± SEM and were analyzed with an independent-sample *t*-test. n = 10 in each group.

signalling and function, which resulted in hyperactivity. Given that development of the striatum undergoes crucial anatomical changes between adolescence and adulthood it is also possible that the adolescent striatum is more susceptible to TLX knockdown in the dDG than the adult. It may also be that silencing of TLX in the juvenile dDG induced a transient effect, and that compensatory mechanisms are taking place during adolescence so that the hyperactive phenotype is overcome during adulthood.

We did not observe an effect of TLX knockdown on anxiety-related behaviours in either late adolescence, or during adulthood. While we previously observed a thigmotaxis phenotype in mice with a spontaneous deletion of TLX both during adolescence and adulthood [33], there was no evidence of this behaviour in the current study using rats with targeted silencing of the gene within the dDG. Furthermore, there was no change in performance by $LV^{TLXShRNA}$ treated rats in the EPM during adolescence or adulthood. This is in contrast to mice with TLX knockout through homologous recombination that have been reported to exhibit an increase in anxiogenic behaviour in the EPM [59]. This discrepancy in findings may be explained by the fact that the vDG is primarily involved in processing and regulation of anxiety-related behaviours [36,66]. In the current study TLX was knocked down only in the dDG of the rats, while in both previous studies showing anxiolytic behaviour in mouse models, TLX was constitutively knocked out [33,59].

We observed an increase in defecation behaviour in the $LV^{TLXShRNA}$ treated group compared to control during adulthood, but not adolescence. However, the LV^{SCR} treated adolescents exhibit an increase in defecation compared to their adult counterparts. This is in line with the fact that the propulsive capacity of the colon is reduced over time and that gastric digestion slows down with age [67]. Though measuring defecation and urination has previously been used as a record of anxiety-like behaviour in rats [68,69], it has been disputed as less reliable than thigmotaxis as they are influenced weight, sex and strain [70,71]. Nonetheless, the behavioural outputs from the anxiety-related tests showed no change in response to TLX knockdown, while the defecation response revealed increased anxiety-like behaviour in the $LV^{TLXShRNA}$ treated group in the stressful environment of the Open Field. This finding is particularly curious in the light of the studies showing a reduction in anxiety-like behaviour in mice with homologous recombination or spontaneous deletion of the TLX gene for both the Open Field test and EPM [57,59]. To the best of our knowledge, the autonomic response to stress in these mouse models has not been measured to date. It would be thus interesting to examine whether the autonomic and behavioural outputs align when TLX expression is absent from birth.

Examination of behaviour during the FST showed that during the first two triads of the 15-min test there were no differences in the frequency of active and passive behaviours between LV^{SCR} and $LV^{TLXShRNA}$ treated rats from either age cohort. Remarkably, during the third triad of the FST, a marked increase in immobility behaviour, coupled with a decrease in swimming behaviour frequency was observed in the $LV^{TLXShRNA}$ treated adolescents compared to the LV^{SCR} treated controls during late adolescence. This is particularly interesting, given that during adolescence the $LV^{TLXShRNA}$ treated rats were hyperactive. On the contrary, adult $LV^{TLXShRNA}$ treated rats exhibited an increase in swimming behaviour compared to their LV^{SCR} treated counterparts. Exposure to the FST has been shown to activate a multitude of systems and circuits including the sympathetic nervous system, the HPA as well as the dopamine- and serotonin- neurotransmitter systems [72]. Furthermore, the depressive-like phenotype induced by FST has been shown to be reversible after administration of various serotonergic agonists, reuptake inhibitors and transporters [50,73,74]. Additionally, serotonin receptors have been implicated in the regulation of the aggressive phenotype of the mice with spontaneous deletion of TLX [75]. Future studies should investigate the serotonin-TLX interaction in terms of mechanism and dynamics throughout development.

There was no effect of TLX knockdown in the dDG of juvenile rats on cognitive performance during either late adolescence or adulthood. We did not observe any differences in the performance of control or TLX-knockdown rats in the large separation task, or in the neurogenesis-associated smaller and more difficult separation task [42]. What is more, in both tasks and both age cohorts, exploration behaviour of the objects was at chance level. It is possible that presence of the virus itself in the dDG of both LV^{SCR} and $LV^{TLXShRNA}$ cohorts of animals rendered the acquisition of this hippocampal-dependent task too difficult. We used contextual and cued fear conditioning to assess the rodents' ability for associative learning and episodic memory recall [76]. With this test, acquisition could be controlled for more robustly and indeed we saw that all animals learned to associate the unconditioned stimulus (US; shock) with the conditioned stimulus (CS; tone; data not shown) during the acquisition phase of the test. However, TLX knockdown did not affect the ability of the animals to recall, during both adolescence and adulthood. This contrasts to the results obtained from mice where TLX was knocked out through homologous recombination [59] and in a study where we previously showed that mice with a spontaneous deletion of TLX displayed impaired performance in contextual fear conditioning during adolescence but not adulthood [33]. However, in a mouse model with conditional deletion of TLX during adulthood, contextual fear conditioning in the absence of TLX was normal [32], corroborating our findings in rats. These data thus suggest that disrupting TLX during early neurodevelopment affects hippocampus-dependent learning and memory as measured by contextual fear conditioning during adolescence but not later in adulthood. They also show that disruption of TLX during juvenile development does not impair learning and memory as measured by this paradigm. The amygdala-dependent cued fear conditioning response also remained the same between LV^{SCR} and $LV^{TLXShRNA}$ treated rats assessed in adolescence and adulthood. A mouse model employing targeted deletion of TLX in adulthood complements the present findings in that no difference in cued fear recall between controls and mice lacking TLX from 8 weeks of age was observed [32]. In mouse models of TLX deletion during early development however, adult and adolescent TLX knockout mice performed poorer than their control littermates in cued fear conditioning [33,59]. It is possible that the lack of effect of TLX knockdown on cued fear recall was due to the small number of tones presented. Here only 3 tones were presented, while others have performed this task using 15 or even up to 50 tones to measure cued fear recall [82]. Taken together, the results of contextual and cued fear conditioning from the various models of TLX deletion imply that in the absence of TLX during early life, key limbic system structures such as the hippocampus and amygdala may develop abnormally and result in impairment in associated behaviours, while the same does not occur as a result of deletion of TLX during adolescence or adulthood. We found no impairment in spontaneous alternations in the Y maze as a result of $LV^{TLXShRNA}$. In mice with a spontaneous deletion of TLX from birth, a decrease in the percent of alternations during adolescence, but not during adulthood, was observed [33]. Likewise, we did not observe any impairment in the performance of adult $LV^{TLXShRNA}$ treated rats on the rewarded or discrete alternation in the Y maze. Despite the crucial involvement of the septal-hippocampal system in this task, other brain areas have also been shown to play a significant role [45,77], which may have compensated for a lack of TLX in the dDG.

5. Conclusions

Our results illustrate changes in behaviours as a result of TLX knockdown in the dorsal hippocampus. TLX is expressed on stem cells [78] and primarily controls the proliferative capacity of NPCs [79]. Although silencing TLX led to reduced cell proliferation in the dDG, it may have not influenced the function of new-born neurons and associated cognitive processes as assessed by the tasks in the current study and in the time frame of the study. The manifestation of behaviours such as hyperactivity and a depressive-like phenotype in adolescence but not adulthood in response to TLX knockdown during the juvenile period suggest that compensatory mechanisms may be taking place during adolescence for these behavioural phenotypes. However, a limitation of the present study is the lack of experimental group where TLX expression has been silenced during adulthood alone. In order to confirm the temporal effect of TLX during development in future studies, it would be important to perform an experiment employing adult lentiviral delivery of TLX shRNA to the dDG of the rat. Future studies investigating the temporal consequences of TLX knockdown on such behaviours are now warranted, including studies examining a sex-dependent effect of TLX during development and adulthood. Conversely, the differential effects of TLX knockdown on swimming behaviour in the FST and defecation behaviour between adolescent and adult rats suggest a potential age-related role for TLX in stress-induced behaviour and in the autonomic-regulated response to a stressful environment. Further investigation may aid deeper understanding of the role of TLX and parallel pathways to it in neurobiological disorders and their potential treatment.

Declarations of interest

None.

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3. Function of hippocampal neurogenesis	yes	No	No	No	Yes	167 x 82.8	9.6

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Born This Way: Hippocampal Neurogenesis across the Lifespan

Kozareva, D.A. [1], Cryan, J.F. [1,2] & Nolan, Y.M.[1]

[1] Department of Anatomy & Neuroscience, University College Cork, Ireland

[2] APC Microbiome Ireland, University College Cork, Ireland

To whom correspondence should be addressed:

*Yvonne M. Nolan, Department of Anatomy and Neuroscience, University College Cork, Ireland

email: y.nolan@ucc.ie

tel: +353 214205476

Summary

The capability of the mammalian brain to generate new neurons through the lifespan has gained much attention for the promise of new therapeutic possibilities especially for the ageing brain. One of the brain regions that maintains a neurogenesis-permissive environment is the dentate gyrus of the hippocampus. Here, new neurons are generated from a pool of multipotent neural progenitor cells to become fully functional neurons that are integrated into the brain circuitry. A growing body of evidence points to the fact that neurogenesis in the adult hippocampus is necessary for certain memory processes, and in mood regulation, while alterations in hippocampal neurogenesis have been associated with a myriad of neurological and psychiatric disorders. More recently, evidence has come to light that new neurons may differ in their vulnerability to environmental and disease-related influences depending on the time during the life course at which they are exposed. Thus it has been the topic of intense research in recent years. In this review we will discuss the complex process and associated functional relevance of hippocampal neurogenesis during the embryonic/postnatal period and in adulthood. We will further consider the literature surrounding hippocampal neurogenesis and its functional role during the adolescent, and the ageing stages of the lifespan with a view to providing insight into the potential of harnessing neurogenesis for health and therapeutic benefit.

Key words: neurogenesis; hippocampus; lifespan; memory; adolescence; ageing

Introduction

The process of generating functional neurons from stem and precursor cells in the central nervous system (CNS) was originally believed to occur strictly during embryonic and early postnatal development in mammals. A century ago this dogma was challenged with the discovery of neurogenesis in the adult brain. Ezra Allen was the first to demonstrate that mitosis persisted in the lateral walls of adult albino rats (Allen, 1918). Several decades later, Altman and Das followed up this research and determined that neurogenesis occurred in the adult rat and guinea pig hippocampus (J. Altman & Das, 1965; Joseph Altman & Das, 1967). However, it was not until the 1990s that the concept of a functional hippocampal neurogenesis began to emerge (Palmer, Ray, & Gage, 1995; Palmer, Takahashi, & Gage, 1997; Suhonen, Peterson, Ray, & Gage, 1996). Since then, evidence has accumulated to demonstrate the existence of this process in the human hippocampus (Boldrini et al., 2018; Eriksson et al., 1998). While controversies still persist, it is generally accepted that neurogenesis occurs in the adult hippocampus and has functional relevance (Jason S. Snyder, 2018).

Animal studies have provided substantial evidence that newly born granule cells in the adult hippocampus are electrophysiologically functional and become integrated in existing neuronal networks (Toni & Schinder, 2015; Henriette van Praag et al., 2002). Behavioural studies suggest that these new granule cells play important roles in certain types of cognitive processing such as spatial learning and memory, and in mood regulation (Balu & Lucki, 2009; Bond, Ming, & Song, 2015; Chunmei Zhao, Deng, & Gage, 2008). Moreover, impaired hippocampal neurogenesis has been reported in neurodegenerative and psychiatric conditions (Balu & Lucki, 2009) and efforts to develop therapeutic strategies that employ the hippocampal NSCs are ongoing. It is becoming

apparent that neurogenic processes or rates may differ at various times during the life course. New neurons may thus differ in their response to environmental influences and disease modifying factors at various times during life, which also has functional implications. Thus, hippocampal neurogenesis and its functional relevance has been the topic of intense research during the last decades. In this review we will focus on the multiple steps of the neurogenic process during the embryonic and postnatal developmental periods, in the hippocampus in adulthood, and also during the other developmentally critical periods of adolescence and older age. We will further evaluate the existing evidence regarding the functional roles of adult-born granule cells at different stages of development with a view to providing insight into the potential of harnessing hippocampal neurogenesis for health and therapeutic benefit.

Embryonic and postnatal hippocampal neurogenesis

In the earliest stage of development, three distinct cell types comprise the embryo – the endoderm, from which the lining of most visceral organs arises; the mesoderm, from which all bones and skeleton muscles develop; and the ectoderm, which gives rise to the entire nervous system and the skin (Bear, 2015). The ectoderm is the outer most layer of the embryo and once it acquires the potential to form neural cells, the process of embryonic neurogenesis begins (Hartenstein & Stollewerk, 2015). For the single sheet of ectoderm to become neurogenic, the following two changes need to take place: firstly, a specialized area of the ectodermal tissue, known as the neural plate, forms a groove; secondly, the walls of this groove or the neural folds move close together, thereby shaping the neural tube (Semple, Blomgren, Gimlin, Ferriero, & Noble-Haeusslein, 2013).

The brain and spinal cord develop from the neural tube, while the peripheral nervous system develops from the neural ectoderm lateral to the neural tube (Bear, 2015). In rodents, the formation of the neural tube is completed at approximately embryonic day (E) 9-9.5, which also marks the start of embryonic neurogenesis (Semple et al., 2013). Neural epithelial cells (NECs), considered as the earliest embryonic NSCs, divide asymmetrically at first, so that they expand their pool while also creating daughter neural progenitor cells (NPCs). Later after the neural tube is completely formed, NECs transform into radial glial cells (RGCs; (J. Zhang & Jiao, 2015)). RGCs also divide asymmetrically to maintain their population and give rise to neurons, glia and potentially other type of cells (Alvarez-Buylla, García-Verdugo, & Tramontin, 2001). RGCs can produce neurons directly or indirectly through NPCs. NECs, NPCs, and RGCs can also divide symmetrically to increase their populations. By asymmetric division, NPCs renew themselves and produce a second daughter cell that either differentiates into a neuron immediately, or goes through a fixed number of divisions before permanently differentiating into a neuron (Alvarez-Buylla et al., 2001; Hartenstein & Stollewerk, 2015). The embryonic stage-dependent NSC differentiation throughout development is depicted in **Figure 1**. RGCs also function as a scaffold through which the newborn neurons and NPCs migrate to their designated area directed by extracellular signals (J. Zhang & Jiao, 2015). In rodents, at approximately E15-E17.5, all neurons comprising the cortical and subcortical areas have been generated and have migrated, which is also when RGCs differentiate into astrocytes and glia (X. Jin, 2016; Semple et al., 2013).

Interestingly, the development of the DG follows another unique path, which may have important consequences regarding the neurogenic permissive environment that is formed there postnatally.

A separate source of progenitor cells (also known as the dentate neuroepithelium; DNE) separates from the neural tube and migrates in close proximity to the pial surface (Urbán & Guillemot, 2014). At E12.5 the hippocampal neuroepithelium and the cortical hem surround the DNE, and at E14.5 the DG becomes clearly distinguishable with dentate precursor cells migrating from the ventricular zone towards the pial surface of the cortex (Rickmann, Amaral, & Cowan, 1987). RGCs from the hippocampal neuroepithelium produce hippocampal neurons and at E17.5 the hippocampal fissure is formed. The dentate precursor cells then begin to migrate and accumulate within the fissure. Some of these cells are thought to comprise the future layer of NSCs of the adult SGZ, while some produce neurons that form the GCL along with neurons produced by the hippocampal neuroepithelium progenitors of the ventricular zone (Urbán & Guillemot, 2014). The typical V-shape of the DG, created by its two blades is contributed by cells produced by the cortical hem (Cajal-Retzius cells), which direct the migration of NPCs and the organization of the hippocampal fissure (Del Río et al., 1997). It is still contested whether the adult NSCs of the SGZ come from the DNE itself at around E13.5 (Seki et al., 2014; Bettina Seri, García-Verdugo, Collado-Morente, McEwen, & Alvarez-Buylla, 2004), or whether they are generated perinatally within the ventral DG (vDG), migrate in late gestation to the dorsal DG (dDG), and become activated through Sonic Hedgehog (Shh)-signalling (Li, Fang, Fernández, & Pleasure, 2013). Thus, the origin of the adult NSCs may have important anatomical and temporal consequences to their function and regulation. Due to the lack of unique immunocytochemically detectable markers specific to NSCs as opposed to NPCs, embryonic NSCs are defined as multipotent self-renewing cells that have the capacity to populate a developing region of the CNS (I.-S. Lee, Jung, Kim, & Park, 2010). Though not exclusive for embryonic NSCs, a large number of studies have identified nestin and SRY (sex

determining region Y)-box 2 (SOX2) as reliable markers of multipotency of NSCs (Ellis et al., 2004; Wiese et al., 2004).

The postnatal period of the rodent is markedly different from that in humans in terms of cellular and system development. For instance, oligodendrocyte maturation (Craig et al., 2003), immune system development (Holsapple, West, & Landreth, 2003) and blood-brain barrier development (BBB; (Engelhardt, 2003)) take place at postnatal day (P) 1-P3 in rodents, while for humans these processes take place prenatally (Daneman, Zhou, Kebede, & Barres, 2010; Dean et al., 2011). In light of these differences, it was recently proposed that to objectively compare neurogenesis across species, neurogenic rates should be reflected as proportion over the lifespan of the species, rather than aligned to age post birth (Jason S. Snyder, 2019). During later postnatal development (between P7 and P10), rodents exhibit their highest brain growth spurt (Bockhorst et al., 2008), highest level of glial cell generation (Catalani et al., 2002; Kriegstein & Alvarez-Buylla, 2009) and growth of axonal and dendritic density (Baloch et al., 2009; Bockhorst et al., 2008). In this period the development of the rodent immune system is consolidated (Holsapple et al., 2003). In the final stage of postnatal development, just before the juvenile period commences at about P20 - P21, the synaptic density in the rodent brain peaks (Micheva & Beaulieu, 1996), being twice as high as the synaptic density observed in adults. Additionally, changes in neurotransmitters and their receptors are also observed (Micheva & Beaulieu, 1996; Romijn, Hofman, & Gramsbergen, 1991).

To date, not much is known about the rate of early postnatal neurogenesis in the rodents. However, it has been shown that within the hippocampus there is a rapid and robust increase of glial fibrillary acidic protein (GFAP) -expressing cells, possibly astrocytes, which reach a peak between P11 and P16 (Catalani et al., 2002). This growth coincides with the rodent critical period of synaptogenesis in the molecular layer of the DG. Up until P4 the synaptic density represents less than 1% of what is observed in the adult brain. At P14 peak levels of synaptogenesis occurs which then levels off at P25 to that consistently observed in the adult hippocampus (Crain, Cotman, Taylor, & Lynch, 1973). This concurrence of events is interesting in the light of findings that astrocytes can modulate neuronal activity in the young rodent brain in response to glutamatergic transmission through the metabotropic glutamate receptor 5 (mGluR5), the expression of which however is significantly decreased after P14 in astrocytes of the rodent hippocampus (W. Sun et al., 2013). This suggests a possible mechanism for controlling neuronal activity driven by asotrcyte-mGluR5-mediated regulation of synaptogenesis. It also suggests that there may be fundamental differences in the neuronal-astrocyte signalling between the immature and adult brains (Semple et al., 2013; W. Sun et al., 2013).

While most of the granule cells of the rodent hippocampus are generated up until P10 (J. Altman & Bayer, 1990; Piatti, Espósito, & Schinder, 2006), RGCs remain abundant in the developing brain until P14 (Malatesta, Hartfuss, & Götz, 2000) when they start differentiating into NPCs. This is followed by their transformation to neuroblasts and finally to mature excitatory granule cells that integrate in the circuitry by P21 (Kriegstein & Alvarez-Buylla, 2009). These changes have been confirmed in nestin-eGFP (green fluorescent protein) transgenic mice whereby the intermediate

filament nestin is used as a marker of neural stem cells. A comprehensive analysis of NPCs in mice aged from P7 and P28 revealed that, not only did the number of NPCs decrease over this developmental period, but also that the genetic profile of the NPCs from the two ages was markedly different and this thus suggests early adulthood senescence (Gilley, Yang, & Kernie, 2011). Furthermore, such results were replicated in a recent study employing single-cell RNA (ribonucleic acid) sequencing and *in situ* hybridization. The data show that the process of neurogenesis in the DG undergoes a transformation from the embryonic to the postnatal period represented by a shift in RGCs around P14 from an embryonic to an adult state. This process was coupled with an increase in NSC differentiation towards neuroblasts rather than glia (Hochgerner, Zeisel, Lönnerberg, & Linnarsson, 2018). While the authors argue that NPCs and neuroblasts sustain very similar properties throughout later stages of life, they have identified a distinct phenotype of the adolescent and adult hippocampal granule cells of the rodent, which will be discussed in detail in the following sections.

Function of hippocampal neurogenesis during embryonic and postnatal development

The function of neurogenesis during embryonic development is to populate the various regions of the CNS with different types of neurons derived from NSCs of the neural tube ((Kandel, Schwartz, & Jessell, 2000); **Table 1, Figure 1**). The hippocampal formation is largely developed by E20 in rodents (Bayer, 1980), and by 20 weeks of gestation in humans (Gómez & Edgin, 2016). However, volumetric development persists to P21 in rodents and to 2 years of age in humans (Ainge & Langston, 2012; Hevner, 2016). The DG is the last hippocampal structure to appear at E16 in rats

with the GCL formed at E21. It undergoes a 50% daily volumetric increase from P1 to P7, and 21% from P7 to P21, when growth and neurogenesis levels level off (Bayer, 1980). The heightened level of neurogenesis during early postnatal days in rodents and the first years in humans has been linked to the phenomenon of infantile amnesia (**Table 1, Figure 3**), or the absence of memory for events that have taken place within the first 2-3 years of life as well as the vague recollections of occurrences from 3 to 7 years of age (Josselyn & Frankland, 2012). The existence of infantile amnesia has been demonstrated across a wide range of tasks such as conditioned suppression (Campbell & Campbell, 1962), passive (Schulenburg, Riccio, & Stikes, 1971) and active (Klein & Spear, 1969) avoidance (Feigley & Spear, 1970), appetitive discrimination (Campbell, Jaynes, & Misanin, 1968), contextual fear conditioning (Rudy & Morledge, 1994; Weber, McNally, & Richardson, 2006), spatial navigation in the MWM (Brown & Kraemer, 1997) and novel object recognition (NOR; (Anderson et al., 2004)). Given that each of these tasks has rather distinct characteristics in terms of the stimuli employed and performance demands, in addition to the consistent results illustrating that memory persistence is proportional to age at the time of acquisition, it has been concluded that infantile amnesia occurs during early postnatal development across species (Josselyn & Frankland, 2012). Direct evidence linking neurogenesis and infantile amnesia came from experiments where neurogenesis was manipulated (inhibited or enhanced) during different points of the lifespan. The results illustrated that increasing neurogenesis promoted forgetting in adult mice, while reducing neurogenesis facilitated memory persistence in infant mice (Akers et al., 2014). To sum up, the function of early life postnatal hippocampal neurogenesis appears to be related to weakening existing memories and information storage in favour of strengthening the ability to learn new things and to acquire new information through rapid continuous generation of large number of new granule cells (Akers et al., 2014; Josselyn &

Frankland, 2012). The adaptive value of this function is considered to be in the rapid clearance of old information that may not be useful, in order to facilitate increased capacity and reduced interference between memories.

Adult hippocampal neurogenesis

Adult neurogenesis encompasses several consecutive phases of development, which are preserved in the adult brain and result in the production of new neurons (Gonçalves, Schafer, & Gage, 2016). However, it is noteworthy that neurogenesis occurs only in the DG, which based on past terminology is not part of the hippocampus proper but rather part of the hippocampal formation, thus what adult hippocampal neurogenesis really refers to is neurogenesis in the adult DG (Gerd Kempermann, 2015). Here, putative hippocampal precursors are multipotent and divide infrequently. Morphologically, they are similar to radial glia with a triangular cell body and a large apical process extending and forming multiple arborizations into the dentate granule cell layer. The end processes of these stem cells terminate onto the vasculature. The progeny of SGZ stem cells migrate to the granule cell layer of the DG where they integrate into hippocampal circuitry as mature excitatory neurons (Gage, 2000).

The first piece of evidence to show the existence of adult hippocampal neurogenesis emanated half a century ago. However, the first direct data set illustrating functional integration of newly born neurons in the adult CNS followed 20 years later from a study of songbirds (Paton & Nottebohm, 1984). After another decade a crucial advance occurred – the introduction of bromodeoxyuridine

(BrdU) to the study of adult neurogenesis (H. G. Kuhn, Dickinson-Anson, & Gage, 1996). BrdU is a synthetic nucleotide analogue to thymidine which incorporates into proliferating cells, and thus allows for investigating their cell fate at later timepoints (Russo et al., 1984). This along with the development of retroviral methods for tracing cells from birth and marking specific genetic markers (Henriette van Praag et al., 2002), in combination with immunohistochemistry (IHC), confocal imaging and electrophysiology allowed for the field of neurogenesis to become one of the most exciting in modern research. The rapid progress during the past decades which was bolstered by the discovery of adult hippocampal neurogenesis in the human brain (Eriksson et al., 1998) has brought about a soaring interest on the topic among the public and scientific community.

Human adult hippocampal neurogenesis: Controversies and convergence

More than two decades ago, the rate of proliferation and the process of functional integration of adult born neurons into the existing circuitry was reported to be remarkably similar across species (Eriksson et al., 1998). However, this has been recently contested, and it is now thought that the rate of generation and maturation of newborn neurons is significantly different between rodents and humans (Jason S. Snyder, 2019). A study measuring the concentration of nuclear bomb test-derived ^{14}C in genomic deoxyribonucleic acid (DNA), found evidence for the birth of as many as 700 new neurons each day in the human hippocampus (Spalding et al., 2013). Nonetheless, the controversy over the existence of human hippocampal neurogenesis has been raised once more (H. Lee & Thuret, 2018; Jason S. Snyder, 2018). In a recent investigation of postmortem brain tissue obtained from 18 adults and 19 perinatal and postnatal samples (age range: 14 gestational weeks to 77 years), it was reported that no newborn neurons were found in the DG of adults and only a few isolated young neurons were observed in samples from young individuals (7 to 13 years of

age). The samples with the most numerous immature neurons observed came from perinatal and postnatal (up to 1 year of age) tissue (Sorrells et al., 2018). On the contrary, using a similar immunohistochemical approach, another group of researchers observed immature and mature adult born neurons in the hippocampal samples obtained postmortem from 28 healthy individuals (age range: 14 to 79 years of age) and the number of each cell type was estimated to be at least in the thousands (Boldrini et al., 2018). It is possible that the big discrepancy in results stems from the fact that in the former study, tissue was obtained from individuals suffering a wide range of diseases (although full medical history was not provided), while in the latter study tissue was obtained from healthy individuals (Jason S. Snyder, 2018). Given the similarity in methods employed, both studies clearly demonstrate the limitations and caveats in studying neurogenesis in human postmortem tissue. Importantly, researchers need to produce detailed reports on the medical records of the patients whose tissue has been examined since factors such as postmortem delay and timing of tissue fixation can have a profound effect on protein degradation, specifically in the case of the fast-degrading doublecortin (DCX), a protein present on immature neurons that has been used as a common marker of neurogenesis (Lucassen et al., 2019). To further address the existence of the phenomenon in the human brain, future studies need to examine not only evidence on an immunohistochemical level but also on a transcriptomic and gene expression level. For instance, single cell sorting and sequencing could aid in profiling the cells and establishing whether indeed granule cells born at different stages of the lifespan have unique characteristics and hence should not be considered as one homogenous population (Jason S. Snyder, 2019). Additionally, an approach for studying hippocampal neurogenesis *in vivo*, has been well characterized but hardly used, namely the use of magnetic resonance spectroscopy where metabolites enriched in stem cells were identified based on their distinct resonance at specific frequency in fatty acids (Manganas et

al., 2007). Hence, a combination of technological approaches could aid in advancing our knowledge of the neurogenic process in humans and reconciling the various data obtained from postmortem human tissue.

The Hippocampus as a Neurogenic Niche

The cells comprising the adult SGZ are the NPCs along with their progeny, immature neurons, glial and endothelial cells, immune cells, microglia and macrophages, and extracellular matrix (Gerd Kempermann, 2015). The only neuronal type known to date to be produced through adult hippocampal neurogenesis is the granule cell (Gerd Kempermann, 2015). It has been proposed that γ-aminobutyric acid (GABA)-ergic inhibitory neurons or basket cells are also generated in the adult rat hippocampus (Liu et al., 2003). The excitatory granule neurons receive input from the entorhinal cortex, exhibit sparse action potentials, are modulated by interneurons in the DG and hilus, and arise from the precursor cells of the SGZ, also known as the neurogenic niche of the hippocampus (Gerd Kempermann, 2015). The term "niche" refers to an anatomical area, wherein the stem cells reside owing to the special microenvironment created by the niche. In the case of the SGZ, it is permissive for neurogenesis and neuronal development to occur (Ming & Song, 2011). The term was first coined in the study of transplantation of hematopoietic progenitors (Schofield, 1978). It has been suggested that such niches are surrounded by a common basal membrane (Mercier, Kitasako, & Hatton, 2002). Since the vasculature is a very important factor for the maintenance and function of the hippocampal neurogenic niche, the term "vascular niche" has also been proposed (Palmer, Willhoite, & Gage, 2000). The NSCs interact closely with the endothelial cells in a multifaceted fashion (Wurmser et al., 2004) and also extend processes, called

"end feet", connecting to the vasculature of the SGZ (Filippov et al., 2003). Additionally, vascular endothelial growth factor (VEGF) has been shown to be a robust modulator of adult hippocampal neurogenesis (K. Jin et al., 2002; Schänzer et al., 2004). Along with the vasculature, the neurogenic permissiveness of the SGZ is brought about due to the distinctive environment of various circulating growth factors, unique extracellular matrix and cell-to-cell contacts, with astrocytes being the most prominent supporting cells for the neurogenic process (Gerd Kempermann, 2015). For instance, astrocytes in the SGZ have been found in very close proximity to proliferating cells and newborn neurons throughout their developing phase (Plümpe et al., 2006; Shapiro, Korn, Shan, & Ribak, 2005). Furthermore, the neurogenic niche of the hippocampus is innervated by neurons from a multitude of brain areas which employ different neurotransmitter systems as well as by commissural tracts from its contralateral side (Gerd Kempermann, 2015).

Stages of Adult Hippocampal Neurogenesis

The process of adult neurogenesis consists of four stages (nomenclature adopted from (Gerd Kempermann, 2015)): a precursor stage, an early survival stage, a postmitotic maturation stage and a late survival stage. The stages can be further divided into six events or transformations that the cells undergo based on evaluating their morphology and protein expression (**Figure 2**; (Gerd Kempermann, 2015; Gerd Kempermann, Jessberger, Steiner, & Kronenberg, 2004; Steiner et al., 2006)). Firstly, the precursor and expansion stages when NSCs go through three continuous progenitor phases characterized by elevated proliferation levels, is followed by the early survival stage when NPCs exit the cell cycle and the number of newborn neurons significantly decreases

due to elimination. Next, the postmitotic stage is characterized by dendritic and axonal outgrowth, synaptogenesis and the establishment of connections. Finally, the late survival stage, marks the integration of new granule cells into the existing circuitry after fine-tuning of their connectivity (reviewed by (Gerd Kempermann, 2015; Gerd Kempermann et al., 2004)). In rodents, the duration from the first to final stage of neurogenesis is thought to last about 7 weeks, while the process from the birth of a new neuron (NSC asymmetric division) to its complete integration in circuitry has been suggested to last 4 weeks (Gonçalves et al., 2016; Ming & Song, 2011).

The precursor stage

During the precursor stage, several different types of NPCs can be observed within the SGZ. They exhibit distinct morphological features but do not represent separate cell populations, rather they emulate different phases or events from the continuum of adult neurogenesis (Gerd Kempermann, 2015). The cells giving rise to adult hippocampal neurogenesis present morphologically and antigenically as radial glia (Type 1), yet some of the markers of embryonic NSCs are absent from the adult hippocampal NSCs, with their soma located to the SGZ and a single process travelling to and branching out within the molecular layer of the DG (J. Zhang & Jiao, 2015). The origin of Type 1 cells was first demonstrated through pharmacological elimination of mitosis within the DG, followed by examination of the phenotype of the first proliferative cells to re-emerge. The cells presented an astrocyte-like radial morphology (B. Seri, García-Verdugo, McEwen, & Alvarez-Buylla, 2001). Subsequently, was established that the cells giving rise to new neurons in the adult SGZ expressed GFAP and nestin (Bettina Seri et al., 2004), which are commonly used

markers of astrocytes (Szymaś, Morkowski, & Tokarz, 1986) and NSCs, respectively (Gilyarov, 2008; Lendahl, Zimmerman, & McKay, 1990; Park et al., 2010). In a further attempt to characterize hippocampal Type 1 cells Filippov and colleagues showed that the nestin+ radial-glia-like cells exhibited an electrophysiological phenotype similar to that of astrocytes (Filippov et al., 2003), namely a passive current and a reversal potential close to the potassium equilibrium potential (Steinhäuser, Berger, Frotscher, & Kettenmann, 1992). Additionally, these cells lacked the expression of the astrocyte-specific marker calcium binding protein β, (S100β) (Filippov et al., 2003). Lastly, the brain lipid binding protein (BLBP) – a well-established radial-glia cell marker (Brunne et al., 2010; Pinto & Götz, 2007) along with the transcription factor SOX2 – a robust marker of embryonic and adult NSCs (Avilion et al., 2003; Rizzino, 2009; Suh et al., 2007) were also found to be expressed by Type 1 cells ((Steiner et al., 2006); **Figure 2**). It is worth noting that the "true stemness" of Type 1 cells or their "unlimited" potential to self-renew has been contested. *In vitro* studies using primary subependymal and hippocampal cultures have found that only cells isolated from the lateral walls of the ventricles exhibited long-term self-renewal capacity and multipotency, while cells isolated from the DG displayed neuro-progenitor rather than a NSC phenotype (Bull & Bartlett, 2005; Seaberg & van der Kooy, 2002). Several years later, however, the Kempermann lab optimized a method of isolation and propagation of NPCs from the adult murine DG, allowing for consistent procurement of hippocampal progenitors which exhibited stem-cell-like properties such as continual self-renewal capacity (Babu, Cheung, Kettenmann, Palmer, & Kempermann, 2007). Nonetheless, findings from *in vivo* studies have not aided in resolving the issue of "stemness" in hippocampal NPCs (Gerd Kempermann, 2011). While Type 1 cells have been proposed to only undergo asymmetric division (Juan M. Encinas et al., 2011), the opposite has been also shown, namely symmetric division and thus unlimited self-renewal of

Type 1 cells (Bonaguidi et al., 2011). These results reflect the heterogeneity of NSCs and NPCs in the neurogenic niche and the importance of establishing common concepts of the neurogenic stages and markers that can be used to delineate them (Gerd Kempermann, 2015). Furthermore, it has been proposed that Type 1 cells are quiescent stem cells that enter the cell cycle upon activation (Lugert et al., 2010). Various factors which transcriptionally regulate or repress inhibitors of the NSC signalling pathways control cell cycle exit and entry of Type 1 cells (reviewed by (Gonçalves et al., 2016)). Taken together, the population of NPCs within the SGZ of the hippocampus is a very heterogeneous one with the quiescent Type 1 radial glia-like NSCs first in the chain of different types of progenitors that propagate the expansion of the NSC pool. It has been debated whether this population of cells exhibit stemness, specifically the capacity to self-renew interminably (Gerd Kempermann, 2011).

The expansion stage

The resulting Type 2 cells arising from the precursor stage display a different morphology and can be further divided into two subcategories – Type 2a and Type 2b. Type 2 cells are the intermediate progenitors of adult born granule neurons with transient amplifying characteristics (Gerd Kempermann, 2015). Their proliferation constitutes the expansion phase of neurogenesis, when the number of NPCs increases dramatically. The main difference between Type 1 and Type 2a cells is in morphology, while the main difference between Type 2a cells and Type 2b cells is in marker expression. Type 1 and Type 2a cells both express nestin, BLBP and SOX2. While Type 1 cells present the radial-glia-like morphology and express GFAP, Type 2a lack the radial

processes and may or may not express GFAP (**Figure 2**; (Steiner et al., 2006)). Additionally, patch-clamp electrophysiological experiments showed that the two populations of cells (radial glia-like stem cells of Type 1 and the transiently amplifying progenitors of Type 2) also displayed different input resistance values (Fukuda et al., 2003). Type 2 progenitors display highly proliferative activity, which has been shown to be positively and negatively modulated by exposure to voluntary wheel running (Kronenberg et al., 2003) and stress (reviewed by (Kino, 2015)), respectively. Additionally, the proliferative activity of Type 2 cells has been shown to intensify as a result of antidepressant intake through serotonin-mediated mechanisms (J. M. Encinas, Vaahtokari, & Enikolopov, 2006). Furthermore, Type 2 cells receive excitatory GABAergic input which has been suggested as a key regulator of their proliferation and differentiation (Tozuka, Fukuda, Namba, Seki, & Hisatsune, 2005; Wang, Kempermann, & Kettenmann, 2005).

Type 2a and Type 2b are considered to be the delineating mark between glial and neuronal fate in adult hippocampal neurogenesis. Specifically, Type 2a cells have the potency to adopt either fate, while Type 2b cells are restricted to a neuronal lineage (Steiner et al., 2006). This is also reflected by their marker expression; Type 2a express mostly glial markers (BLBP, SOX2, nestin and in some cases GFAP) and Type 2b cells express the pro-neuronal transcription factors neurogenic differentiation 1 (NeuroD1) and prospero homeobox protein 1 (PROX1) as well as the polysialated neural cell adhesion molecule (PSA-NCAM) and the neuronal migration protein DCX (reviewed by (J. Zhang & Jiao, 2015)). The transition from Type 2a to Type 2b cells has been suggested to occur in a gradient-like manner such that the existence of a mixed phenotype of Type 2ab cells has also been proposed (J. Zhang & Jiao, 2015). It is driven by a delicately orchestrated repression and

activation of transcription factors which regulate the fate of the cells in the neurogenic niche. This cascade of events (activation/repression of transcription factors) is conducted through epigenetic mechanisms, neurotransmitter signalling, as well as through growth factor and morphogen release within the neurogenic niche (Faigle & Song, 2013). An example of the gradient expression of different transcription factors on Type 2a and 2b cells is illustrated by the fact that SOX2 and TLX (the human homologue of the Drosophila *tailless* gene) are strongly expressed in Type 1 and Type 2a cells, and their downregulation co-occurs with the upregulation of the T-box transcription factor Eomes (Tbr2), PROX1 and NeuroD1, with the latter marking the transition of the progenitor to a Type 2b NPC phenotype (Faigle & Song, 2013). Studies of constitutive and conditional knockout mouse models have shown that adult neurogenesis cannot persist in the absence of these transcription factors (Brulet, Zhu, Aktar, Hsieh, & Cho, 2017; Christie et al., 2006; Hodge et al., 2008, 2012; Karalay et al., 2011; Young et al., 2002). Type 2b cells also mark the start of DCX and PSA-NCAM expression, markers which are maintained from the expansion and proliferation stage of the neurogenic process through the exit of cell cycle, early maturation and survival stage of the newborn granule cells (**Figure 2**; (Couillard-Despres et al., 2005; J. Zhang & Jiao, 2015)). It is worth noting that despite it being dispensable for neuronal development during adult hippocampal neurogenesis (Merz & Lie, 2013), DCX is conventionally used as a surrogate marker for adult neurogenesis (Gerd Kempermann, 2015).

The early survival and postmitotic maturation stage

Type 3 cells, also known as slowly proliferating neuroblasts, further contribute to the expansion phase but significantly less so than Type 2 cells (Dokter & von Bohlen und Halbach, 2012). Nevertheless, under pathophysiological conditions such as experimentally induced seizures, the proliferative activity of Type 3 cells is dramatically increased (Jessberger, Römer, Babu, & Kempermann, 2005). Type 3 cells cease to express nestin, but they retain DCX, PSA-NCAM, NeuroD1 and PROX1 expression and also continue to receive excitatory GABAergic input (**Figure 2**; (Gerd Kempermann, 2015)). Type 3 cells initiate a short distance migration from the SGZ to the GCL where they exit the cell cycle and undergo a morphological developmental transition, such that they extend processes in a vertical direction. This marks the transition to the early postmitotic maturation phase of the neurogenic process (Dokter & von Bohlen und Halbach, 2012; Gerd Kempermann, 2015).

Promptly upon exit of the cell-cycle, Type 3 cells display expression of the post-mitotic neuronal nuclei (NeuN) marker and the Ca^{2+} -binding protein calretinin, which is only transiently expressed during the early, but not late survival phase of the neurogenic process (Brandt et al., 2003; J. Zhang & Jiao, 2015). The colocalization of BrdU and NeuN is a useful tool for examining survival rate of newborn neurons, by varying the time points of investigation post BrdU administration (C. Zhao, Toni, & Gage, 2013). It has been shown that the number of BrdU+NeuN+ cells is highest during the early post-mitotic phase after BrdU administration, and dramatically decreases over the next days into the late survival phase due to an apoptotic elimination process (Biebl, Cooper, Winkler, & Kuhn, 2000; Gerd Kempermann, 2015). The mechanisms regulating the survival versus apoptosis of a neuroblast within the GCL remain elusive, but various candidates such as

neurotransmitters (Tashiro, Sandler, Toni, Zhao, & Gage, 2006), growth factors (Pfisterer & Khodosevich, 2017), B-cell lymphoma 2 (Bcl-2; (H. Georg Kuhn et al., 2005)), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB; (Imielski et al., 2012)) and tumour protein p63 (p63; (Cancino et al., 2013)) are proposed to be involved. The neuroblasts that survive the initial apoptotic elimination proceed with dendritic development and axon extension towards the CA3 region, where synapses with target cells are formed (G. J. Sun et al., 2013). The axons of adult-born granule cells become part of the mossy fibre tract and significantly contribute to its plasticity and size, both of which have been correlated with the rate of adult neurogenesis (Römer et al., 2011). During this stage, glutamatergic synaptic input begins to innervate the cells and they switch to having inhibitory GABA input once there is sufficient glutamatergic input or once the granule cells adopt glutamatergic phenotype (Tozuka et al., 2005). At this stage the apoptotic elimination is significantly reduced, with a small decrease in newborn neurons two weeks post mitosis (Gerd Kempermann, 2015). Retroviral labelling of adult-born neurons, which allows fluorescent tagging of neurons undergoing mitosis at the time of virus administration, has revealed that axons extend to CA3 around 10 days after the cell has been labelled, while dendritic spines only begin to emerge 17 days post mitosis (Chunmei Zhao, Teng, Summers, Ming, & Gage, 2006).

The late survival and maturation stage

The final stage of adult neurogenesis encompasses the late maturation phase of the newborn neurons. This phase takes place around 3-4 weeks post mitosis when the cells cease to express the immature neuronal markers DCX and PSA-NCAM, while they retain the expression of NeuN,

PROX1 and NeuroD1 and switch their Ca^{2+} -binding protein from calretinin to calbindin (**Figure 2**; (Brandt et al., 2003; J. Zhang & Jiao, 2015)). Though at this phase cells appear to be morphologically mature and fully integrated into the existing circuitry, they still exhibit different electrophysiological properties from mature neurons, namely that the newborn granule cells are a lot more excitable with a significantly lower threshold for long-term potentiation (LTP) induction than their mature counterparts (Schmidt-Hieber, Jonas, & Bischofberger, 2004; Henriette van Praag et al., 2002). This enhanced synaptic plasticity is normalized or inhibited by the local interneurons at approximately 1-1.5 months post mitosis, when the late maturation phase is assumed to conclude as the adult-born granule cell becomes indistinguishable from a granule neuron that has been there since birth (Ge, Yang, Hsu, Ming, & Song, 2007; Marín-Burgin, Mongiat, Pardi, & Schinder, 2012).

Function of hippocampal neurogenesis during adulthood

Adult neurogenesis in the SGZ has been implicated in spatial learning and memory (Gould, Beylin, Tanapat, Reeves, & Shors, 1999; Gerd Kempermann, 2008; Ming & Song, 2011), and in mood regulation ((Levone, Cryan, & O'Leary, 2015; O. F. O'Leary & Cryan, 2014); **Table 1, Figure 3**). Studying the causal link between neurogenesis and cognitive function has been enabled through the utilization of various ablation techniques such as irradiation, pharmacological interventions (with antimitotic drugs to decrease, or antidepressants to enhance neurogenesis), and transgenic mice (reviewed by (Chunmei Zhao et al., 2008)).

Adult hippocampal neurogenesis and cognition

While some inconsistencies are evident from reports through the years, rodent studies have primarily shown that adult hippocampal neurogenesis is necessary for hippocampal-dependent tasks which require spatial and contextual memory as well as for pattern separation (**Table 1, Figure 3**). Targeting the transiently proliferating progenitors or Type 2 cells with an antimitotic drug in adult rats resulted in impaired performance in trace conditioning task involving an eye-blink response (a hippocampal-dependent learning task; (T. J. Shors et al., 2001)), but not in other hippocampal-dependent tasks such as contextual fear conditioning and spatial learning in the Morris Water Maze (MWM) paradigm (Tracey J. Shors, Townsend, Zhao, Kozorovitskiy, & Gould, 2002). Upon conditional deletion of TLX in the hippocampi of adult mice, which led to reduced NPC proliferation and thus reduced neurogenesis, mice exhibited impaired spatial learning and memory in the MWM task, but no change in performance on contextual fear conditioning, which is another form of hippocampal-dependent learning (C.-L. Zhang, Zou, He, Gage, & Evans, 2008). Interestingly, two other methods of neurogenesis ablation - irradiation and genetic ablation of NPCs - produced different results from those described above. Specifically, reducing neurogenesis with these techniques in adult mice resulted in their failure to learn the association between a given context and receiving an electric shock (fear conditioning), while there was no deficit in their performance in the MWM task or the Y maze (Saxe et al., 2006). In addition, localized X-irradiation to the hippocampus and cortex in adult mice resulted in impaired learning in the Barnes maze task, but not the MWM task, both of which are hippocampal-dependent tasks measuring spatial memory and learning (Raber et al., 2004). What is more, adult rats that underwent irradiation also performed poorly on the fear conditioning paradigm (Winocur,

Wojtowicz, Sekeres, Snyder, & Wang, 2006) and place learning (measured by T-maze task), but not in MWM task or NOR task (Madsen, Kristjansen, Bolwig, & Wörtwein, 2003). Interestingly, when neurogenesis was suppressed with an antimitotic drug, adult rats showed impaired memory in the NOR test (Bruel-Jungerman, Laroche, & Rampon, 2005). No effect of irradiation was observed on MWM task performance or in the novelty suppressed feeding test in adult mice (Meshi et al., 2006). On the contrary, after receiving a low dose of irradiation, adult rats displayed impaired long-term spatial memory as measured by the MWM (J. S. Snyder, Hong, McDonald, & Wojtowicz, 2005). It is interesting to note that some ablation techniques led to impaired learning of certain types of tasks, but not others. These results point to the complexity of the function of adult born neurons and also to the fact that the specific stage of neurogenesis which is targeted by the intervention can influence the specific cognitive task (Chunmei Zhao et al., 2008). Furthermore, differences in the specific tests used as well as the species and strains may additionally account for the discrepancies in results. Interestingly, the treatment of brain cancer in humans often requires cranial radiation therapy which has been associated with progressive cognitive decline such as impairments in memory, attention and executive function (Sarkissian, 2005). These side effects have been attributed in part to the decrease in hippocampal neurogenesis that the treatment may cause (Greene-Schloesser, Moore, & Robbins, 2013). Behavioural studies in rodents where neurogenesis is enhanced through enrichment have shown positive correlation between the level of adult hippocampal neurogenesis and performance on hippocampal-dependent tasks such as MWM and pattern separation (reviewed by (Chunmei Zhao et al., 2008)).

Pattern separation refers to the ability to form distinct representations of similar inputs or the process of disambiguating those similar inputs by producing dissimilar outputs (Treves, Tashiro, Witter, & Moser, 2008). This computational process has been suggested to play role in the discrimination between similar memories and to be dependent on the newly generated granule cells in the DG formation (Aimone et al., 2014; Clelland et al., 2009; Deng, Aimone, & Gage, 2010; J. S. Snyder et al., 2005). Behavioural tests evaluating pattern separation processing in rodents focus on testing the capacity of animals to discriminate between similar spatial or context-dependent (e.g., olfactory, auditory) stimuli that demand different behavioural responses (França, Bitencourt, Maximilla, Barros, & Monserrat, 2017). Fear conditioning paradigms, novel object location recognition paradigm, radial arm maze paradigm, or touchscreen operant conditioning paradigms have been used in this regard (van Hagen, van Goethem, Lagatta, & Prickaerts, 2015; Yassa & Stark, 2011). Despite many findings supporting the involvement of hippocampal neurogenesis in pattern separation (Hvoslef-Eide & Oomen, 2016), discrepancies in the literature still exist (Cushman et al., 2012; Groves et al., 2013). Nonetheless, after performing a systematic review and meta-analysis of studies using ablation of hippocampal neurogenesis to test its involvement in behavioural pattern separation, and using effect sizes rather than statistical significance (p-value) as a metric method of evaluating the compatibility between results of different studies, França and colleagues found that the majority of data consistently supported a strong reliance of pattern separation on hippocampal neurogenesis (França et al., 2017). Moreover, enhancing neurogenesis through voluntary exercise for instance was coupled with enhanced performance on the spatial pattern separation paradigm novel object location and in NOR task in mice ((Bolz, Heigele, & Bischofberger, 2015; Creer, Romberg, Saksida, van Praag, & Bussey, 2010); **Table 1**). A study where hippocampal neurogenesis was decreased using a lentiviral

approach, demonstrated direct dose-dependent involvement of immature neurons and brain-derived neurotrophic factor (BDNF) action in the consolidation of similar memories ((Bekinschtein et al., 2014); **Table 1**). To sum up, notwithstanding some discrepancies in the research thus far, a potential role of hippocampal adult-born granule cells in learning and memory appears to be important for at least some types of memory processing.

Adult hippocampal neurogenesis and mood regulation

As well as being involved in learning and memory, adult neurogenesis has been shown to play a role in mood regulation, and in particular in antidepressant action (Duman, Nakagawa, & Malberg, 2001; Malberg, Eisch, Nestler, & Duman, 2000; Santarelli et al., 2003; Tanti & Belzung, 2013; Warner-Schmidt & Duman, 2006). In addition, as depression is widely associated with stress-related disorder, it is worth noting that adult hippocampal neurogenesis also plays a key role in buffering stress responses in animals (Mirescu & Gould, 2006; Petrik, Lagace, & Eisch, 2012). For instance, it has been extensively characterized (see for instance (Naninck et al., 2015)) that exposure to stressors early in life may have profound effects on the different stages of hippocampal neurogenesis and susceptibility to anxiety and depression later in life (reviewed by (Fitzsimons et al., 2016; Lucassen et al., 2015)). Patients suffering from major depressive disorder have presented with reductions in hippocampal volume, which may be reflective of reductions in neurogenesis (Malykhin, Carter, Seres, & Coupland, 2010). However, it is worth noting that evidence to position neurogenesis as an etiological factor in the development of mood disorders is lacking due to the fact that ablation of neurogenesis does not induce depressive-like or anxiety-like behaviours in

rodents in the absence of another negative stimulus such as a stressor (Petrik et al., 2012; Chunmei Zhao et al., 2008). It is now two decades since Duman and colleagues showed that antidepressants from a variety of classes (including electroconvulsive therapy, tranylcypromine, imipramine, lithium and fluoxetine) have been shown to increase the number of newborn granule cells in rodent models of depression (Malberg et al., 2000). Such findings were bolstered by studies using genetic and radiological methods for selectively ablating hippocampal neurogenesis that demonstrated that the behavioural effects of the antidepressants fluoxetine and imipramine on the novelty suppressed feeding test (a behavioural paradigm shown to be dependent on hippocampal neurogenesis) were prevented in adult mice, as was the anti-anxiolytic effect of fluoxetine in a chronic unpredictable stress paradigm ((Santarelli et al., 2003); **Table 1**). In contrast, the antidepressant-like effects of non-monoaminergic based antidepressant-like drugs, such as CRF1 or V1b receptor antagonists, are not affected by inhibition of adult hippocampal neurogenesis (Bessa et al., 2009; Surget et al., 2011) suggesting a dissociation between the role of neurogenesis in mediating the behavioural effects of different types of treatments. In human post-mortem brain tissue there is also evidence that hippocampal progenitor cell proliferation is increased with antidepressant medication in depression (Boldrini et al., 2012). Findings from rat studies appear to be more consistent in their demonstration of the necessity of newly generated granule cells for antidepressant effectiveness. This is apparent in the forced swim test (FST) and novelty suppressed feeding test in response to X-ray irradiation-based ablation of neurogenesis (Jiang et al., 2005) and the chronic stress-induced decrease in neurogenesis-paradigm ((Airan et al., 2007); **Table 1**). Nonetheless, the precise mechanism and function of hippocampal neurogenesis in antidepressant-mediated mood regulation remains to be elucidated with many controversies arising from different species and methods used by different labs (for reviews see (Petrik et al., 2012; Chunmei Zhao et al., 2008)).

Other functions of adult hippocampal neurogenesis

With respect to the potential functions of adult hippocampal neurogenesis in the human brain, the most we have learned to date comes from postmortem examination of patients who suffered from CNS disorders. Nevertheless, magnetic resonance spectroscopy has provided a useful tool for imaging NPCs in the live human brain under physiological and pathological conditions (Manganas et al., 2007). With this we have learned that neurogenesis is altered in cases of epilepsy such that seizures cause a dramatic increase in the proliferation of hippocampal NPCs and Type 3 neuroblasts which is associated with cognitive deficits (reviewed by (Lindvall & Kokaia, 2015); (Jessberger & Parent, 2015)). Furthermore, enhanced neurogenesis has been shown to follow ischemic stroke in rodents, and newborn neurons are also suspected to repopulate infarct areas in human patients (Lindvall & Kokaia, 2015; Chunmei Zhao et al., 2008). Lower levels of proliferation in the hippocampus were demonstrated in adult patients suffering from schizophrenia. Moreover, the impaired cognitive function in schizophrenic patients resembles the impairments observed when hippocampal neurogenesis is inhibited (Reif et al., 2006). What is more, genetic studies suggest that the decrease in newborn cells might be important contributing factor to the development of the disease. A recent meta-analysis suggested that the connection between neuropsychiatric disorders and dysregulated hippocampal neurogenesis is beyond epiphenomenon, and that adult-generated granule cells may hold the key to therapeutic and/or preventative strategies for alleviating suffering from these disorders (Yun, Reynolds, Masiulis, & Eisch, 2016). The processes contributing to deregulation of hippocampal neurogenesis under pathological conditions are not fully understood, but it is proposed that extracellular signals

provided by the hippocampal microenvironment may interact with cell-intrinsic factors, which could present potential drug targets for the conditions listed above (Ryan & Nolan, 2016).

Hippocampal Neurogenesis during vulnerable periods of the lifecourse

Adolescent hippocampal neurogenesis

The levels of neurogenesis in the adolescent rodent hippocampus are much higher compared to adults as illustrated by a mouse study comparing the number of BrdU+ and DCX+ cells between mice at age P30 and mice at age P120. There was a 4-fold decrease in the number of proliferating (BrdU+) NPCs and the number of immature neurons (DCX+) from adolescence to adulthood suggesting an adolescent-associated increase in plasticity coupled with a dramatic reduction in neurogenesis during the transition from adolescence to adulthood (He & Crews, 2007). The mechanisms by or purpose for which this occurs are poorly understood and only a limited number of studies to date have focused on investigating the process of hippocampal neurogenesis during adolescence. Interestingly, it has been shown that the process and time course of apoptosis of newborn granule cells in the adolescent DG closely reflects that of the adult DG, albeit to an exaggerated degree (Curlik, Difeo, & Shors, 2014). Specifically, juvenile rats (P21-P23) were administered an intraperitoneal (i.p.) injection of BrdU and the number of cells, which incorporated BrdU was analysed one- and three-weeks post injection. It was found that more than 7 000 proliferating cells retained BrdU expression within a week. However, most of them were no longer detected 3 weeks after the injection, indicating a similar rate of apoptosis as in the adult hippocampus (Curlik et al., 2014; Epp, Spritzer, & Galea, 2007). Findings from our lab have

illuminated an age-specific effect of voluntary exercise on hippocampal neurogenesis such that, adolescent-initiated running led to an increased expression of a wide array of plasticity- and neurogenesis-related genes in the hippocampi of Sprague Dawley (SD) rats compared to rats that had access to a running wheel during adulthood. Among the upregulated genes were the pro-neurogenic *bdnf*, *tlx* and *dcx*, and the pre- and post- synaptic regulating genes *synaptophysin* and *psd-95* (postsynaptic density protein 95; (J. D. O'Leary, Hoban, Cryan, O'Leary, & Nolan, 2019)). Moreover, in another cohort of SD rats comparing effects of voluntary exercise initiated either during adolescence or adulthood, we showed that both the number and complexity (measured by number of neurites, their length and branch points) of DCX+ cells were significantly increased in the hippocampi of rats exercising since adolescence compared to their respective controls. When exercise was initiated during adulthood, hippocampal DCX+ cells presented with increased complexity, but not number, compared to the non-exercising controls (J. D. O'Leary et al., 2018).

Much research has concentrated on investigating the effects of alcohol exposure during adolescence on brain development and cognitive behaviour (reviewed by (F. T. Crews, Vetreno, Broadwater, & Robinson, 2016)). Interestingly, it has been consistently shown that hippocampal neurogenesis is significantly reduced in adolescent and adult rodent models of binge drinking. However, while neurogenesis recovers to normal levels in adults after a period of abstinence, when the alcohol exposure took place during adolescence rather than adulthood the deficit persists until late adulthood (F. Crews, He, & Hodge, 2007). Such a long-lasting reduction in hippocampal neurogenesis was also observed after administration of alcohol to adolescent rhesus monkeys (Taffe et al., 2010). Furthermore, binge ethanol exposure during adolescence has been shown to

directly influence NSCs and NPCs but not neuroblasts, by reducing proliferation and increasing apoptosis in cells of both the dDG and vDG of rodents (rats; (Vetreno & Crews, 2015)) and non-human primates (rhesus monkeys; (Taffe et al., 2010)). This was also coupled with impaired cognitive performance on memory tests (Taffe et al., 2010; Vetreno & Crews, 2015). Moreover, during the abstinence period in adolescent rodents, neuroblasts were ectopic, and were found in the molecular layer of the DG rather than within the SGZ (McClain, Morris, Marshall, & Nixon, 2014). Collectively, these studies not only highlight the sensitivity of the adolescent brain to positive environmental factors such as voluntary exercise and negative ones such as alcohol exposure, but also emphasize that altered hippocampal neurogenesis during adolescence may be an important factor which underpins susceptibility to changes in hippocampal-dependent cognitive function in later life. Further research will delineate the functional relevance of hippocampal neurogenesis to environmental influences during adolescence.

Function of hippocampal neurogenesis during adolescence

Several studies have investigated whether inhibiting hippocampal neurogenesis during adolescence results in similar impairments as observed when the process was inhibited in adulthood (**Table 1, Figure 3**). For instance, cranial irradiation of the rat hippocampal region during pre-adolescence resulted in a dramatic increase in apoptosis and impaired production and release of growth factors in the hippocampus, while the same procedure performed in adulthood resulted in sustained release of pro-inflammatory cytokines in the hippocampus (Blomstrand, Kalm, Grandér, Björk-Eriksson, & Blomgren, 2014). Chronic stress induced a transient reduction

in the number of proliferating NPCs in the hippocampi of adolescent but not adult male mice suggesting a resilience by adolescent mice to impairments induced by the stress. This phenomenon could not be accounted for by the damage to emotional processing and sociability caused by the inhibition of hippocampal neurogenesis since both adolescents and adults exhibited normal performance on depression-related behavioural tests as well as a regular corticosterone response after acute exposure to stress (Kirshenbaum, Lieberman, Briner, Leonardo, & Dranovsky, 2014). Interestingly, using the same method of transient ablation of hippocampal neurogenesis, another group of researchers found the same outcome of memory and anxiety-related behaviours for both adolescent and adult female mice. However, impaired female-female social interaction resulted when neurogenesis was inhibited during adolescence but not adulthood, reflected by a complete social aversion towards conspecifics, as well as impaired pup retrieval (Wei, Meaney, Duman, & Kaffman, 2011). We have also shown that social isolation stress during adolescence can lead to impaired exercise-induced increased neurogenesis in the hippocampus (Kozareva, O'Leary, Cryan, & Nolan, 2018). Furthermore, in a study comparing the effect of the antidepressant fluoxetine administered during adolescence and adulthood on hippocampal neurogenesis and serotonin synthesis in rats, it was shown that treatment with fluoxetine during adolescence but not adulthood increased neurogenesis and serotonin synthesis in the vDG but not dDG (Klomp, Václavů, Meerhoff, Reneman, & Lucassen, 2014). Additionally, adolescent- versus adult- initiated voluntary exercise in rats had differential effects on performance on cued- and context- dependent fear conditioning with adult-initiated exercise enhancing performance on both tasks without influencing the expression of neurogenic and plasticity markers, while adolescence-initiated exercise did not change performance on the fear conditioning tasks but enhanced expression of neurogenesis and plasticity markers (J. D. O'Leary et al., 2019). Our research has further

demonstrated that adolescent- but not adult-initiated exercise in rats was associated with an increase (albeit transient) in performance on pattern separation in touchscreen-based task coupled with increased neurogenesis (J. D. O'Leary et al., 2018). Interestingly, a stronger positive correlation between the neurite length of new neurons and cognitive flexibility as measured by reversal learning on a touchscreen-based task, was observed in response to the adolescent compared to adult-initiated exercise (J. D. O'Leary et al., 2018). Moreover, a greater degree of complexity in the new neurons in the hippocampus of rats exposed to exercise during adolescence compared to adulthood was reported (J. D. O'Leary et al., 2018).

Such comparisons of treatment and ablation outcomes between adolescence and adulthood, however, need to be considered in the context of not only differences in the basal levels of neurogenesis across development, but also in terms of the hormonal and behavioural changes that occur during the adolescent period. Though limited in number, rodent studies have consistently shown that ablation of neurogenesis during adolescence results in decreased proliferation and survival of hippocampal NPCs from adolescence until late in adulthood, which also correlates with a persistent impairment in performance on memory-related tasks such as fear conditioning and the MWM test (Achanta, Fuss, & Martinez, 2009; Rola et al., 2004). Similarly, human studies of children and adolescents with cancer who have had to undergo radiation therapy, have shown that such treatment is associated with lasting changes in intelligence quotient (IQ) scores and cognitive performance (Rodgers, Trevino, Zawaski, Gaber, & Leasure, 2013). Defective hippocampal neurogenesis during adolescence has been suggested as a contributing factor to the onset and development of psychiatric disorders (reviewed by (Hueston, Cryan, & Nolan, 2017)), which in

combination with the fact that adolescence is a period of dramatic vulnerability to the effect of extrinsic influences, means that it is imperative to expand our understanding of how positive and negative regulators of hippocampal neurogenesis such as stress and exercise influence the brain during this critical period.

Hippocampal neurogenesis during ageing

Hippocampal neurogenesis is presumed to persist throughout the lifespan, however, a decline in neurogenesis has been recognized to occur with age across species. In fact Altman and Das (1965) in their pioneering paper commented on the decrease of cell birth within months after birth (J. Altman & Das, 1965; Gerd Kempermann, 2015; Klempin & Kempermann, 2007). The first report to quantify age-related changes in adult hippocampal neurogenesis came from a study of 12- to 21-month old rats where the authors showed, through BrdU labelling and IHC analysis immediately after, or 4 to 6 weeks after BrdU administration, that a decrease in mitotic activity of NPCs in the SGZ occurred and was associated with a net decrease in neurogenesis (H. G. Kuhn et al., 1996). Furthermore, Kempermann and colleagues showed a similar age-related decrease in hippocampal neurogenesis in 8- to 20-month old mice, and the decrease in neuronal survival could be somewhat ameliorated by enriched housing conditions (G. Kempermann, Kuhn, & Gage, 1998). The age-associated decrease in hippocampal neurogenesis has also been shown in tree shrews. This study also demonstrated that older animals were more susceptible to a stress-induced decline in NPC proliferation than their younger counterparts (Simon, Czéh, & Fuchs, 2005). Interestingly, despite a net decrease in hippocampal neurogenesis in wild-living aged squirrels and chipmunks,

it was shown that there was a species difference in terms of the age-related decrease observed. Specifically, the number of proliferating NPCs was decreased in the DG of squirrels, while the number of immature adult born neurons was diminished in the DG of chipmunks (Barker, Wojtowicz, & Boonstra, 2005). This finding is particularly interesting, in light of highlighting the complexity of studying the neurogenic process in non-captive populations, since the squirrels were relying on neurogenesis-dependant strategies (spatial memory) to locate their hidden food stores, while the chipmunks had much less developed spatial memory and relied on a single place for food (Barker et al., 2005). Aside from rodents, it has been shown using BrdU incorporation, that hippocampal neurogenesis persists in non-human primates, namely the Macaque monkeys, until they are 23 years old (the human equivalent of old age). However, the rate of neurogenesis occurred at significantly lower levels than during adolescence and adulthood (Gould, Reeves, et al., 1999). Similar to what has been reported on adult human hippocampal neurogenesis, this existence of an age-related decline in neurogenesis remains controversial (Jason S. Snyder, 2018). Intriguingly, the researchers who propose the occurrence of neurogenesis in the adult human hippocampus, have not found a decline of NPC proliferation or of neurogenesis with age (Boldrini et al., 2018; Eriksson et al., 1998), despite a reported age-associated decline of the quiescent progenitors pool (Boldrini et al., 2018).

The mechanisms underlying the age-related decline in hippocampal neurogenesis remain poorly understood. It has been proposed that within the senescent brain the neurogenic niche may be deprived of the extrinsic signals regulating the neurogenic process or that the aged NPCs are less responsive to normal signalling within the niche, or both (Gerd Kempermann, 2015). The evidence

accumulated thus far points to changes in the properties of the neurogenic niche with age, rather than changes in the phenotype of the NS/PCs themselves. For instance, it has been reported that the numbers of NSCs and NPCs as well as the proportion of astrocytes to neurons in the hippocampus of young and aged rats remained the same, however, there was a decrease in the number of cells actively undergoing mitosis in the aged animals (Hattiangady & Shetty, 2008). The authors speculated that this was due to changes in the milieu of the neurogenic niche based on their earlier observations that important regulators of neurogenesis such as BDNF and CREB (cyclic adenosine monophosphate (cAMP)- response element binding protein) decreased dramatically in the DG of middle-aged and aged rats (Hattiangady, Rao, Shetty, & Shetty, 2005). Additionally, it was shown that the gradual loss of hippocampal neurogenesis in aged mice was associated with downregulation of the mitotic factor survivin in a Wnt-dependent signalling manner (Miranda et al., 2012). This finding was corroborated with the observation that the Wnt antagonist Dickkopf-1 increased with age, while mice deficient in Dickkopf-1 not only exhibited enhanced hippocampal neurogenesis during aging, but also performed better at neurogenesis-dependent tasks, involving spatial working memory, than age-matched controls whose Dickkopf-1 expression was not modulated (Seib et al., 2013). Growth factors such as epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1), which are important regulators of adult neurogenesis have also been shown to play a role in the age-related decline of hippocampal neurogenesis in the rodent (reviewed by (Gerd Kempermann, 2015)). For instance, intracerebroventricular (i.c.v.) administration of basic fibroblast growth factor (FGF-2) and EGF resulted in not only a reversal in age-related decrease in neurogenesis, but also an enhancement of the number of adult born neurons in the aged hippocampus, illustrating that the aged brain is still susceptible to the influence of exogenous growth factors (K. Jin et al., 2003). Moreover, the levels

of FGF-2 as well as IGF-1 and VEGF, were found to dramatically decline in the hippocampi of aged rats (Shetty, Hattiangady, & Shetty, 2005). Additionally, this FGF-2 decline was as a result of an age-related deterioration of FGF-2 synthesis by astrocytes, leading to a reduced number of GFAP+FGF2+ radial-glia-like cells in the DG of aged rats (Shetty et al., 2005). It was also independently demonstrated that the hippocampus is one of the regions of the rat brain with the highest and most robust expression of the FGF-2 receptor FGFR2, specifically on astrocytes, and that the expression of this protein decreased significantly with age (Chadashvili & Peterson, 2006). Infusion of IGF-1 through i.c.v. ameliorated the decrease of hippocampal neurogenesis in aged rats (Lichtenwalner et al., 2001), while in a model of long-lived mice (the Ames dwarf mice) enhanced hippocampal neurogenesis coupled with increased levels of IGF-1 were observed during aging (L. Y. Sun, Evans, Hsieh, Panici, & Bartke, 2005). Another prominent perpetrator of the age-related decline in hippocampal neurogenesis has been proposed to be the family of glucocorticoid hormones and receptors, the release and circulation of which coincidentally increase with age (Cameron & McKay, 1999). Glucocorticoids have been linked to increased hippocampal atrophy and to regulate adult hippocampal neurogenesis (Egeland, Zunszain, & Pariante, 2015; Odaka, Adachi, & Numakawa, 2017; Sapolsky, 2000). Another piece of evidence supporting the impairment in the neurogenic niche properties over time stems from a study where aged mice were infused with vascular and neurogenic factors of young mice which resulted in a rejuvenated neurogenic niche and a restoration of hippocampal neurogenesis (Katsimpardi et al., 2014). Furthermore, despite the lack of alterations in properties of hippocampal Type 1 and Type 2 cells with age, a group of researchers illustrated through IHC labelling of cells with BrdU and NeuN a possible delayed maturation of adult born neurons in the aged DG (Rao, Hattiangady, Abdel-Rahman, Stanley, & Shetty, 2005). Taken together, these findings highlight the complex

interplay of different factors within the neurogenic niche that may be affected by mechanisms driven through the ageing process and which thereby ultimately affect the number of immature neurons produced in the aged brain.

Function of hippocampal neurogenesis during ageing

Adult hippocampal neurogenesis has been proposed to be a key element in ensuring and maintaining functional hippocampal integrity in old age (Gerd Kempermann, 2015; Gerd Kempermann, Gast, & Gage, 2002). Neurodegenerative diseases due to the age-dependent rapid and continuous loss of neurons (such as Parkinson's and Huntington's disease), have been suggested to reflect the contraposition of the neurogenic process such that under homeostatic conditions a fine balance between neurodegeneration and neuroregeneration exists, and under pathological conditions the balance is disturbed and a disease manifests (Gerd Kempermann, 2015). Even though little evidence has accumulated in support of the abovementioned theory, if deemed true, it in combination with findings regarding the high potential of stem-cell-based strategies for the treatment of age-related neurodegeneration and pathologies, make the hypothesis that adult neurogenesis holds a key to novel therapeutic approaches in the treatment of age-related neurodegenerative disorders rather attractive (Lindvall & Kokaia, 2015; Lindvall, Kokaia, & Martinez-Serrano, 2004). In addition, decreased hippocampal neurogenesis is proposed as an important mechanism underlying age-related cognitive decline as well as neurodegenerative disorders such as Alzheimer's disease and various types of dementia (Kuzumaki et al., 2010).

Nonetheless, the mechanisms of how hippocampal neurogenesis can function as therapeutic target for neurodegenerative conditions, remain to be further elucidated.

Similar to studies on adult hippocampal neurogenesis, the function of hippocampal neurogenesis in rodents during ageing has been studied using neurogenesis enhancing and ablating techniques in conjunction with neurogenesis-associated cognitive tasks in aged animals under normal physiological conditions (**Table 1, Figure 3**). Given the positive correlation between physical activity and the reduced risk of dementia and cognitive decline in an elderly cohort (Laurin, Verreault, Lindsay, MacPherson, & Rockwood, 2001), the Gage lab investigated whether hippocampal neuroplasticity may account for the cellular mechanism underpinning these observed benefits using a rodent study approach. To test their hypothesis, the authors exposed middle aged mice (10 months old) to an enriched environment, consisting of a rearrangeable set of plastic tubes, a running wheel, and nesting materials and toys for the duration of the 10 month study, a period in mice considered to reflect senescence in humans (Gerd Kempermann et al., 2002). Interestingly, the mice exposed to the enriched environment displayed a fivefold increase in the number of newborn neurons compared to controls, which was coupled with significant enhancements of their learning and memory performance on the MWM task, as well as exploratory behaviour in an open field (OF) task and locomotor activity on the rotarod. This suggests that living in a stimulating environment during ageing can induce an increase in hippocampal neuroplasticity and cognitive performance (Gerd Kempermann et al., 2002). In a study employing neurogenesis-associated behavioural tests, which probed spatial memory and pattern separation in aged rats, the authors found a positive correlation between structural alterations and neurogenesis in the hippocampus,

and performance on the behavioural tests (Driscoll et al., 2006). Specifically, with the advancement of age, rats displayed decreased hippocampal volume and hippocampal neurogenesis, which was paralleled by impairments in cognitive performance on the MWM task and a pattern separation paradigm (Driscoll et al., 2006). More recently, a study in mice examined the effects of senescence on the different stages of hippocampal neurogenesis on both learning and spatial memory performance on the MWM task. The results illustrated that the decline in neurogenesis over time could best be modelled by an exponential inverted U-shape curve, such that the most rapid decline occurred between 3 and 6 months of age, after which neurogenic levels slowly but steadily decreased. Interestingly, the decrease could be accounted for in all stages of the neurogenic process, namely proliferation, differentiation and survival. What is more, the authors found that performance in the MWM task was progressively worse with age not due to impairments in learning, but due to mice adopting more spatially imprecise strategies over time (Gil-Mohapel et al., 2013). As well as decreased neurogenesis, neurodegenerative diseases are also characterized by neuronal loss primarily due to apoptosis (Lunn, Sakowski, Hur, & Feldman, 2011). Upon examination of the effect of genetically induced hippocampal neuronal loss in the ageing mouse brain, it was found that apoptosis had an enhancing effect on hippocampal neurogenesis. However, despite proliferation and neuronal differentiation and survival being significantly increased, these transgenic animals performed significantly poorer on spatial memory in the Barnes maze. The results illustrated that the increase in the number of granule cells did not mitigate the cognitive deficit observed with ageing (Yeung et al., 2014). Interestingly, human depressed patients exhibited decreased numbers of progenitor cells, which contrary to findings from rodents, albeit adult not aged, did not increase in number in response to antidepressant treatment (Lucassen, Stumpel, Wang, & Aronica, 2010). Thus, neurogenesis alone cannot account

for the age-related cognitive decline observed in rodents and humans, and more mechanisms need to be taken into account for successful development of preventative and therapeutic strategies to ameliorate the deterioration of cognitive function during senescence (Gerd Kempermann, 2015).

Conclusion

The field of hippocampal neurogenesis has become firmly established over the last decades and research of this topic has significantly expanded our knowledge and understanding of the properties of NSCs, the stages of the neurogenic process and the potential functional roles of newly generated granule cells. The impact on our perspective of brain plasticity and its potential under pathological and physiological conditions has fostered current studies to continuously examine and manipulate NSCs to further explore their regenerative capacity. However, significant challenges still remain. Firstly, the evidence accumulated across species needs to be reconciled in terms of the differences in rate at which neurogenesis occurs, the different stages of the lifespan at which neurogenesis peaks and the functional consequences of such species-specific variations. For instance, the approach for studying neurogenesis in humans needs to be adapted to the challenges presented by working with human samples. Secondly, the heterogeneity of the neurogenic niche needs to be acknowledged and further evaluated using techniques such as single-cell sequencing that would help us better define the properties of the cells during different stages of the process as well as the changes that newly-born granule cells undergo between birth and full maturation. Lastly, reconciling the role of neurogenesis in hippocampal function remains to be achieved. For this, studies need to employ more selective, inducible and reversible manipulations of the

neurogenic process *in vivo*. To conclude, this review highlights autonomy of hippocampal neurogenesis across adolescence, adulthood and ageing in different species. Neurogenesis in the adult rodent brain has already been extensively characterized. Now, using this knowledge in conjunction with new technologies will bring us closer to understanding the process of hippocampal neurogenesis across the lifespan in rodents as well as humans and to assimilating ways in which it can be used for improving brain health.

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Table 1: Function of hippocampal neurogenesis through the lifespan

Intervention	Function of neurogenesis	Species	Reference
Embryonic & Early Postnatal development			
IHC, volumetric & morphologic analysis	Populate the hippocampal formation with neurons	Rodents (by E20) Humans (by gest. week 20)	(Bayer, 1980; Gómez & Edgin, 2016)
	Complete volumetric development of the DG	Rodents (P21) Humans (2 years old)	(Ainge & Langston, 2012; Hevner, 2016)
Inhibition/Enhancement of neurogenesis & behavioural interventions	Weakening existing memories and information storage in favour of strengthening the ability to learn new things and to acquire new information (infantile amnesia)	Shown across species	(Akers et al., 2014; Josselyn & Frankland, 2012)
Adolescence			
Behavioural interventions & inducing increase in neurogenesis (exercise)	Affiliative behaviour	Mice	(Wei et al., 2011)
	Processing of stress-inducing stimuli (social defeat; social isolation)	Mice	(Kirshenbaum et al., 2014; Kozareva et al., 2018)
Inducing increase in neurogenesis (fluoxetine)	Response to antidepressant treatment of vDG newborn neurons	Rats	(Klomp et al., 2014)
Ablation of neurogenesis through irradiation during early life/adolescence	Impaired fear conditioning and MWM performance in adulthood	Rats & Mice	(Achanta et al., 2009; Rola et al., 2004)
	Impaired IQ scores and cognitive performance	Human	(Rodgers et al., 2013)
Impaired neurogenesis	Implications in psychiatric disease	Rodents	Reviewed by (Hueston et al., 2017)
Adulthood			

Ablation of neurogenesis through irradiation	Impaired fear conditioning but not spatial memory (MWM, Y-maze)	Mice	(Saxe et al., 2006)
	Impaired spatial learning & memory in Barnes maze but not MWM	Mice	(Raber et al., 2004)
	Normal spatial learning and memory (MWM) and anxiety-like behaviour (novelty suppressed feeding test)	Mice	(Meshi et al., 2006)
	Impaired pattern separation (radial arm maze & touch screen – for similar but not distinct spatial locations)	Mice	(Clelland et al., 2009)
	Blocked antidepressant-induced enhanced behavioural performance and neurogenic levels	Mice	(Santarelli et al., 2003)
	Impaired fear conditioning and place learning (T-maze), but normal MWM and NOR	Rats	(Madsen et al., 2003; Winocur et al., 2006)
	Impaired long-term spatial memory (MWM)	Rats	(J. S. Snyder et al., 2005)
	Blocked pharmacologically-induced enhanced behavioural performance and neurogenic levels	Rats	(Jiang et al., 2005)
	Brain cancer treated with cranial radiation therapy associated with cognitive decline (impaired memory, attention and executive function)	Human	(Greene-Schloesser et al., 2013; Sarkissian, 2005)
Pharmacological ablation of neurogenesis	Impaired ability to acquire trace memories, but not fear memories or perform in the MWM (spatial memory) & EPM (anxiety-like behaviour)	Rats	(T. J. Shors et al., 2001; Tracey J. Shors et al., 2002)
	Impaired memory in NOR	Rats	(Bruel-Jungerman et al., 2005)
Transgenic/knock down methods for ablation (each study has targeted different genes)	Impaired spatial learning and memory (MWM), but normal fear conditioning	Mice	(C.-L. Zhang et al., 2008)
	Impaired pattern separation (radial maze for similar but not distinct spatial locations)	Mice	(Clelland et al., 2009)
	Blocked antidepressant-induced enhanced behavioural performance and neurogenic levels	Mice	(Santarelli et al., 2003)
	Increased anxiety-like behaviour (EPM)	Mice	(Revest et al., 2009)

	Impaired spatial learning, but not memory (MWM)	Mice	(X. Zhao et al., 2003)
	Impaired spatial memory consolidation, but not learning	Mice	(M. Zhao et al., 2007)
	Impaired spatial learning and memory (MWM)	Mice	(Shimazu et al., 2006)
	Impaired pattern separation (recognition memory for similar but not distinct locations)	Rats	(Bekinschtein et al., 2014)
Enhancement of neurogenesis through learning and/or enrichment	Classic study illustrating that hippocampal-dependent associative learning enhances (doubles) the number of adult-born neurons in the hippocampal formation	Rats	(Gould, Beylin, et al., 1999)
	Enhanced long-term memory in NOR	Rats	(Bruel-Jungerman et al., 2005)
	Enhanced learning (MWM) and long-term potentiation	Mice	(H. van Praag, Christie, Sejnowski, & Gage, 1999)
	Enhanced long-term pattern separation (recognition memory for similar objects in NOR)	Mice	(Bolz et al., 2015)
	Enhanced long-term pattern separation (recognition memory for similar locations in NOL)	Mice	(Creer et al., 2010)
Pharmacological enhancement of neurogenesis	Enhanced anti-depressant effects on novelty suppressed feeding test and enhanced anti-anxiolytic effect in chronic unpredictable stress paradigm	Mice	(Santarelli et al., 2003)
	Anxiolytic and anti-depressant like behaviour performance (FST & novelty supressed feeding test)	Rats	(Jiang et al., 2005)
Transgenic methods for enhancement	Enhanced neurogenesis but no change in hippocampal-dependent learning and memory	Mice	(Morcuende et al., 2003)
Observational studies	Decreased hippocampal volume in patients with major depressive disorder (positively affected by long-term treatment with antidepressants)	Human	(Malykhin et al., 2010)

	Lower levels of proliferating cells post-mortem found in hippocampi of schizophrenic, but not depressed patients	Human	(Reif et al., 2006)
	Level of neurogenesis across different mouse strains correlates with learning, but not memory performance (MWM)	Mice	(G. Kempermann & Gage, 2002)
Ageing			
Enhancement of neurogenesis through learning and/or enrichment	Enhanced learning and memory performance (MWM) & hippocampal-independent behaviours (locomotion, exploration) Exercise enhanced learning and memory consolidation in MWM	Mice	(Gerd Kempermann et al., 2002) (Henriette van Praag, Shubert, Zhao, & Gage, 2005)
Observational studies	Impaired learning & memory (MWM & pattern separation) Impaired learning & memory (MWM) in a dose-dependent manner with reduction in neurogenesis	Rats	(Driscoll et al., 2006) (Drapeau et al., 2003)
	Impaired learning & memory in MWM due to imprecise adoption of search strategies, correlated with reduced neurogenesis	Mice	(Gil-Mohapel et al., 2013)

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Figure Captions

Figure 1: Embryonic neurogenesis.

During early gestation (E9-E9.5 in rodents) stem cells (light blue) self-renew and expand their pool to generate progenitors of the different neural cell lineages. During mid-gestation (E15 in rodents) the neural stem cells produce neuronal progenitors (dark blue), which continue asymmetric division to populate the CNS with neurons (red cells). The gliogenic phase begins when glia progenitors (yellow) generate mainly astrocytes (orange). During late gestation (E17.5 in rodents), the oligodendrocytic progenitors (dark purple) give rise to mainly oligodendrocytes (light purple) and some astrocytes.

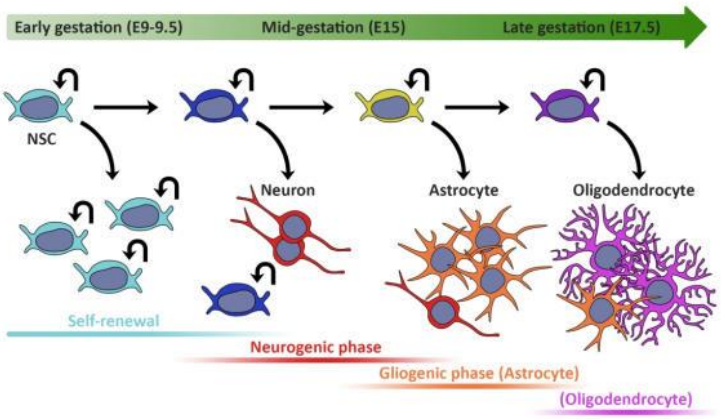
Figure 2: Stages of hippocampal neurogenesis.

Depiction of the stages of the neurogenic process in the hippocampus. The radial glia-like stem cells (Type 1; blue) maintain their pool through self-renewal and give rise to progenitor cells expressing similar markers but displaying different morphology (Type 2 (A&B); green), which undergo rapid proliferation and begin to express markers specific to the neuronal fate of their progeny. Type 2 cells generate neuroblasts (Type 3; yellow). The neuroblasts enter the early survival stage (orange cells) and extend processes towards the molecular layer. During the late survival stage, only newborn neurons that have formed functional connections and have matured morphologically (red cells) remain from the thousands of neuroblasts generated. Granule neuron somata are represented in purple. The colour-coded bar on top illustrates the gradual transition in

marker expression as the cells progress through the different stages of the neurogenic process. The grey-gradient-scale bar on the bottom represents the switch of newborn neurons from GABA to glutamatergic input. ML: molecular layer; GCL: granule cell layer; SGZ: subgranular zone.

Figure 3: Function of hippocampal neurogenesis

Function of hippocampal neurogenesis through the lifespan as evidenced by literature summarized in **Table 1**.



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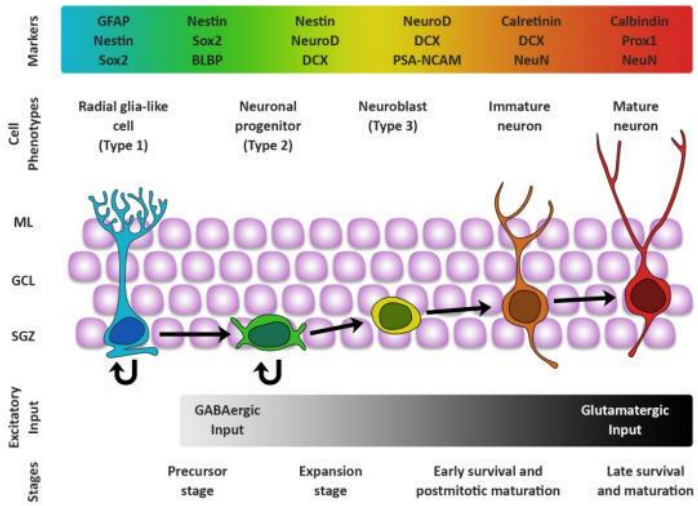

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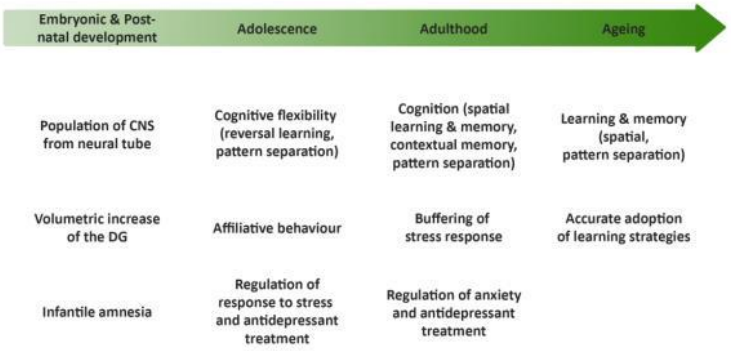

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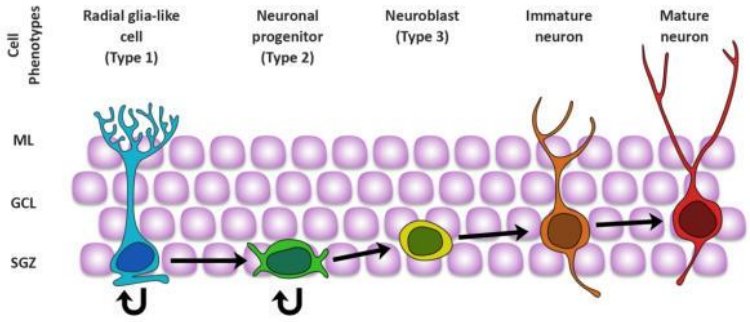

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